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PREFACE

We regret that difficulties beyond our control have led to the small size of the present volume of this *Review*, and it is our hope that the quality of these chapters will make up in part, at least, for the deficit. Exigencies of time and various duties have forced postponement to Volume 16 of scheduled reviews on the effect of specific antibodies on tissue cells, recent trends in food processing, metabolism of microorganisms as related to their pathogenicity, and viral genetics. These reviews, together with those scheduled for next year, will give rise to a volume more truly reflecting the growing literature of microbiology.

We wish to extend our thanks to the authors of the present volume for their labors in behalf of microbiologists in general who will be aided, possibly either by active or passive transport processes analogous to those reviewed herein for microorganisms, in obtaining further insight into the vagaries and complexities of microbic life. We are pleased to present our first review by a Russian author and it is our hope that there will be an ever increasing exchange of ideas between all peoples.

Our thanks are extended to Dr. Wayne Umbreit for his valuable aid in the past five years. Our newest member, Dr. Boris Magasanik, is welcomed as the successor to Dr. Umbreit in the continuing pattern of change in the Editorial Committee.

The untimely death of Dr. Jerome Syverton is recorded here with deep regret. Dr. Perry E. Treadwell has agreed to co-operate with Dr. John D. Ross in completing the review which had been started by Dr. Syverton and Dr. Ross.

Again, as in past years, we express our appreciation for the assistance and co-operation of the editorial staff and of Banta Company, and, in particular, acknowledge our gratitude to Miss Beryl V. Daniel for her capable efforts and encouragement.

C.E.C.	B.M.
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PHYSIOLOGY OF ACTINOMYCETES[1,2]

By Vincent W. Cochrane
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Introduction

Two reviews in 1953, those of Gottlieb (91) and of Perlman (183) were devoted to this topic, and the present review, with few exceptions, is not designed to cover work done before 1953. Since that time, special topics, especially antibiotics, have been reviewed several times; the subject is also considered in chapters of a general work on the actinomycetes by Waksman (252), and in conjunction with the physiology of fungi by Cochrane (38).

Some topics, traditionally classifiable as physiology, have had to be omitted, to the extent that only about half the 1953–1960 literature is cited. Structures of antibiotics are not covered unless there is information as to their biosynthesis; nor are cultural conditions for antibiotic formation reviewed. Other topics omitted include the effects of radiation, actinophage and lysogeny, metabolites of undetermined nature, and the ecology of actinomycetes. Although the physiologist cannot ignore our developing knowledge of genetics, I will refer here only to the recent symposium edited by Szybalski (240) as a convenient summary of current knowledge.

The space given to each topic is in large part determined by the volume of published work on that topic. It will be evident that some physiological problems have received relatively little attention, others having been perhaps overemphasized. In general, it is not unfair to suggest that study of the physiology of actinomycetes has lagged behind that of other bacteria; there is a tendency to do just enough work on a given problem to satisfy oneself that the actinomycetes have the same mechanism as other microorganisms for a given function. It will be necessary to go further than that, if only to explain the ecological peculiarities of the group, and much further if the mechanisms of biosynthesis are to be understood.

Taxonomy of the actinomycetes is confused, and the physiologist hesitates even to comment on a field in which the angels have so clearly feared to tread. A newcomer to work on actinomycetes might with some justice be cautioned to identify the actual culture he uses clearly enough so that further work can, if necessary, be done with the same culture, regardless of its formal taxonomic designation at the moment.

Composition

The cell wall.—Avery & Blank (1) corrected earlier views by showing that neither chitin nor cellulose, characteristic of the fungi, occurs in actinomy-

[1] The survey of literature pertaining to this review was concluded in December, 1960.

[2] The following abbreviations are used: DAP (diaminopimelic acid); DPN (diphosphopyridine nucleotide); RNA (ribonucleic acid); TPN (triphosphopyridine nucleotide).

cetes. Lysozyme sensitivity and the formation of osmotically sensitive structures analogous to bacterial protoplasts have been shown by Bradley (21) and by Douglas, Robinson & Corke (62) in *Streptomyces* spp. Nickerson and associates (201, 203, 231) demonstrated lysozyme sensitivity in most, but not all, isolates of *Streptomyces*, while cell walls of *Nocardia* spp. were uniformly not affected by the enzyme. A hexosamine was detected as a major component in *Streptomyces* spp., and may be the lysozyme substrate; *Nocardia* isolates were low in this component.

A polysaccharide, probably of cell wall origin, isolated by Bishop & Blank (15) from *N. asteroides*, was found to have a branched structure and to yield on hydrolysis arabinose and galactose in the molar ratio 1.7:1.

Recent work by Cummins & Harris (49, 50), Davis & Baird-Parker (52), Davis & Freer (53), and by Hoare & Work (115), the first two papers cited being based on studies of purified cell walls, throw light both on cell wall composition and on relationships within the Actinomycetales. Strains assigned to *Actinomyces* fall into two groups: one, referable to *A. israeli* and almost all of human origin, is characterized by the presence of lysine and the absence of DAP in hydrolysates of the cell wall. Other strains described as *A. bovis* form an inhomogenous assemblage with respect to wall composition and do not resemble the first group. *Streptomyces* is characterized by LL-diaminopimelic acid as the predominant or perhaps only isomer of DAP, and by the absence of lysine. *Propionibacterium* (four of five strains) closely resembles *Streptomyces*. Typical *Nocardia* isolates differ from *Streptomyces* and resemble *Mycobacterium* in that they yield on hydrolysis DL-DAP and arabinose. However, in *Nocardia*, isolates which fit the classical picture of the genus may not show the same cell wall composition (53). *Micromonospora* isolates have DL-, LL-, and (possibly) DD-DAP, with no detectable lysine; muramic acid is present.

Cummins & Harris (49) generalize their findings as they affect ideas on phylogeny. First, the actinomycetes show no relation to the fungi in the composition of their cell walls; this is corroborative of the current view that the Actinomycetales have their closest affinities to the Eubacteriales. Second, relationships with the Eubacteriales are evident also in the overall pattern of amino acids and sugars, the occurrence of DAP or lysine, but not both, and the presence of muramic acid. Third, the actinomycetes, judged from cell wall composition, fit into three natural groups: (*a*) *Mycobacterium*, *Nocardia*, and *Corynebacterium;* (*b*) *Actinomyces* (in part); (*c*) *Streptomyces*, *Micromonospora*, and (possibly) *Propionibacterium*.

Krassil'nikov & Kalakutskii (147) suggest, from microscopic observations, that the cell walls of the aerial mycelium in *Actinomyces* (*Streptomyces*) spp. may contain lipid.

The slime surrounding the cells of *Streptomyces alboflavidus* has been investigated by Pfennig (188), and shown to consist of two fractions. One is a polyglucose, the other yields on hydrolysis many of the components, e.g., DAP, hexosamine, amino acids, and sugars, which characterize cell wall

hydrolysates. Such extracellular sheaths are common in *Streptomyces* [Pfennig (188a)].

Nitrogenous constituents.—Analyses of the free amino acids of the mycelium of *Streptomyces* spp. have been reported by Bekhtereva (5), Bezborodov (9), and by Pfennig (187). Both absolute amounts and relative proportions of the free amino acids are affected by culture age and by the growth medium used. Reusser, Spencer & Sallans (199) determined the presence of amino acids essential for animal nutrition in an isolate of *Streptomyces.*

Phosphorus compounds.—Guberniev and associates (96, 97) determined for *Actinomyces* (*Streptomyces*) *aureofaciens* the distribution of phosphorus in nucleic acids, volutin, and polyphosphates. The polyphosphates appear to fall into three groups: acid-insoluble forms associated with volutin, other acid-insoluble polyphosphates, and an acid-soluble fraction.

Lipids.—Clark & Aldridge (35) demonstrated fat-staining bodies in young mycelium of *Nocardia corallina*. These disappear later and then reappear in old cultures. Their metabolic significance remains to be determined. In *N. asteroides* a new lipid, nocardic acid, has been described by Michel, Bordet, & Lederer (170), and in the same species a lipoprotein fraction has been isolated as a wall or membrane constituent [Guinard, Michel & Lederer (98)]. Kwapínski & Merkel (150) isolated from *S. griseus* mycelium a number of the common fatty acids. A search for sterols carried out by Fiertel & Klein (73) revealed traces in *Micromonospora* sp., no detectable sterol in *S. griseus.*

Growth

Di Marco and co-workers (12, 54, 56) describe in some detail the growth of *S. aureofaciens*. Two phases are distinguishable: a phase of rapid growth and synthesis of proteins and nucleic acids; and a second phase of slower growth toward the end of which the relative amount of protein and RNA actually decreases. Since this relative decline in protein and RNA occurs before autolysis has begun, it is supposed that the cause is an increased synthesis of, for example, cell wall material. Boretti *et al.* (18) and Scotti & Zocchi (216) show that cells from the two growth phases are morphologically distinguishable, mycelium in the second being distinctly thinner. The cycle is changed markedly, however, by supplementation of the complex medium with inorganic phosphate, and antibiotic synthesis is much reduced (12, 54).

In general, most species of *Streptomyces* show growth phases; the phases have been especially fully described for *S. rimosus* by Doskočil *et al.* (60, 61) and by Zaitseva & Orlova (280); for *S. griseus* by Hockenhull (116) and by Inoue (126); and for *Actinomyces* (*Streptomyces*) *aureofaciens* by Prokof'eva-Bel'gorskaya & Popova (195). Although, as a rule, antibiotic production occurs after the bulk of growth has occurred, exceptions to this rule have been demonstrated by Frommer (74) with *S. purpurascens* and by Smith (228) with *S. niveus.*

Two types of influence on growth phases should be cited. Both Hockenhull (116) and Inoue (126) show that change from one complex medium to another changes the pattern of growth and may change the number of stages discernible. More specifically, phosphate has several effects. In *Actinomyces streptomycini* (*S. griseus*), Brinberg & Graborskaia (24) found that phosphate in high concentrations reduced streptomycin yield and accelerated carbohydrate utilization. The phosphate effect on *Actinomyces* (*Streptomyces*) *rimosus* is believed by Zaitseva & Mikhailova (279) to be exerted primarily by suppression of the second growth phase. It seems likely that for both *S. rimosus* and *S. aureofaciens* it is the exhaustion of phosphate which terminates the first phase of growth and initiates the second (61, 280). As will be mentioned later, it has been suggested that phosphate controls the relative contributions of the Embden-Meyerhof and the phosphogluconate oxidation pathways of respiration.

Marshall & Alexander (164) used oxygen uptake as the index of early growth; the cube root of the oxygen uptake plotted against time gave the most nearly linear expression of growth in *Nocardia* sp., *Streptomyces* sp., and several fungi.

Autolysis is usually quite extensive in cultures of *Streptomyces* spp., although exceptions to this rule occur in, for example, the species studied by Sackmann (204). Data secured by Corum *et al.* (45) on *S. erythreus* show the usual autolysis and extensive loss of nitrogen into the medium. In *S. venezualae*, Gottlieb (91) showed in addition that even young cells lose ammonia to the medium. Autolysis is reported by Heim & Lechevalier (106) to be delayed by the provision of calcium; barbital and related compounds also appear, according to the work of Ferguson, Huang & Davisson (72), to delay autolysis.

Cytochemical observations made by Giolitti and others (87, 88) suggest that the aerial mycelium of an agar colony is richer in nucleic acids and in certain enzymes than is the vegetative (substrate) mycelium; presumably, the aerial mycelium has generally greater metabolic activity. Cultures which have lost the capacity to form aerial mycelium are reported by Dondero & Scotti (59) to regain this ability when co-cultured with other species; the induced capacity is non-heritable.

Gottlieb (91) found that spores of *S. venezualae* swell in distilled water but that an energy source is required if germination is to proceed to completion.

Carbon and Energy Metabolism

Carbon nutrition.—Our knowledge of carbon nutrition in the actinomycetes has not changed in principle in the period covered by this review, although many more species have, of course, been studied.

Two actinomycetes have been described in recent years as autotrophic. Ware & Painter (259) isolated an organism able to grow (visual estimate) aerobically on silica gel with cyanide as the sole source of both carbon and

nitrogen. Takamiya & Tubaki (241) describe *Streptomyces autotrophicus* as making very slow growth on a salt medium with hydrogen as the source of energy and carbon dioxide as the source of carbon.

Claims for autotrophic growth require careful verification especially because of the ability of many species of *Nocardia* and *Streptomyces* to grow with hydrocarbon impurities in laboratory air as the source of energy and carbon dioxide as the source of carbon. This phenomenon, oligocarbophily, has been reviewed recently by Hirsch & Engel (113), and demonstrated experimentally by Hirsch (110, 111). Hirsch & Engel (113) point out that claims for carbon monoxide oxidation by actinomycetes are based on inadequate evidence and should not be accepted without further study.

Hydrolysis of polysaccharides.—Evidence predating the period of this review and summarized by Waksman (252) establishes that the actinomycetes as a group are active in the breakdown of cellulose, starch, and other polysaccharides in soil and of the mucopolysaccharide, chitin. Veldkamp (249) and Jagnow (128a) confirm the generalization with regard to chitin.

Jeuniaux (130, 131) showed that an extracellular chitinase is produced by *Streptomyces* spp. in a chitin medium. The enzyme appears free in the culture fluid in quantity only after most of the chitin substrate has disappeared. A centrifugally homogeneous preparation was resolved by electrophoresis into three components, all of about the same specific activity but acting synergistically on chitin. The extracellular chitinase of *S. griseus* was found by Reynolds (200) to be formed only in a chitin medium; the isolated products of its action were N-acetylglucosamine and N,N-diacetylchitobiose. Further studies by Berger & Reynolds (7) show that *S. griseus* forms three enzymes; two of these are chitinases and form from chitin the two products just mentioned; the third is a chitobiase.

The extracellular cellulase of *Streptomyces* sp. has been studied in some detail by Norkrans & Rånby (180), with the conclusion that it acts by random scission of the cellulose chain. Reese, Smakula & Perlin (198) come to the same conclusion regarding another species. The preparation they used accumulates cellotriose and cellobiose, i.e., lacks β-glucosidase activity; however, cellobiose is utilized by intact cells.

The amylase of five species of *Streptomyces* is reported by Simpson & McCoy (226) to be formed only in media containing starch—or, in one instance only—maltose. Like the amylase of *Aspergillus oryzae*, the enzyme is stabilized toward heat by calcium, and this and other properties suggest that the *Streptomyces* enzyme is an α-amylase (209, 226).

Sørensen (232) found, in *S. albus* and *M. chalcea*, an extracellular xylanase, not formed in a glucose medium. The enzyme acts on xylan by random scission; the final products are xylan and xylobiose, the latter evidently split only within the cell. The *S. albus* preparation contains two electrophoretic components with identical enzyme activity. Simpson (225) also reports an extracellular pentosanase in five isolates of *Streptomyces*.

Mannisidostreptomycinase (*α-mannosidase*).—The enzyme-splitting man-

nisidostreptomycin acts, according to work of Hockenhull *et al.* (117), on α-phenylmannoside and appears in the culture fluid in late stages of growth of *S. griseus*. Kollár (144) reports that the enzyme is not, in fact, formed at all until late in the growth process and suggests that it is an inducible enzyme; both α-methyl- and α-phenylmannoside act as inducers under specific conditions of aeration, pH, and temperature. Inhibition by aldohexoses is reported by Maruta & Tanaka (168).

Gross respiration.—There have been no systematic studies of endogenous respiration, but some observations incidental to other work may be noted. Kollár (144) found substantial inhibition of respiration in *S. griseus* by cyanide and arsenate, some by azide; arsenite appeared to be stimulatory. In the same species, Inoue (126) reports an inhibition of respiration in young cells by, surprisingly, streptomycin; Boretti & Raggi (19) show a similar effect of aureomycin on respiration of young cells of *S. aureofaciens*. Pettkó, Kiss & Krámli (186a) found several elements—Cd, Co, Pb, and Zn—inhibitory, and Fe and Mn stimulatory, to respiration in *S. aureofaciens*. Endogenous respiration of *S. griseus* is reduced by starvation, increased by phosphate [Hockenhull *et al.* (118)]. Finally, the rate of endogenous respiration in *S. nitrificans* according to the work of Schatz *et al.* (212), is strongly affected by the medium in which cells are grown.

Cells of *M. vulgaris* respiring in a complete medium show inhibited respiration with pure oxygen and with sulfhydryl poisons; it is suggested by Webley (260) that the oxygen effect may be exerted by inactivation of sulfhydryl enzymes.

Douglas & San Clemente (63), working with *S. scabies*, found that glucose-grown cells oxidize succinate only after a lag; it is possible that this reflects time required for development of a transport mechanism, since the lag is absent in succinate-grown cells. Organic acids are oxidized more rapidly at pH 5.5 than at 7.3 (77).

Endogenous respiration in *S. griseus* is greatly increased by 2,4-dinitrophenol, as shown by Hockenhull *et al.* (118); presumably, although azide is not effective, this indicates control of respiration by mechanisms involving oxidative phosphorylation.

It is the usual experience to find that actinomycetes do not oxidize substrates to completion, and it is likely that this reflects oxidative assimilation. Midwinter & Batt (171), working with *Nocardia corallina* and labeled substrate, show that both propionate and glucose are assimilated in part; the label from assimilated propionate comes out of the cell (as CO_2) readily on continued incubation, that from assimilated glucose, less readily. This suggests two different types of assimilate. Acetate is 50 per cent assimilated under normal conditions, but azide and 2,4-dinitrophenol abolish assimilation.

The genus *Streptomyces* is usually assumed to be strictly aerobic, although Henssen (108) describes two species as facultative. Strict aerobiosis would not necessarily preclude, however, an anaerobic metabolism in the absence of

growth, as is found in some fungi. Experiments with adequate methods, i.e., complete anaerobiosis, carried out by Hockenhull *et al.* (118) indicate in *S. griseus* an appreciable endogenous fermentation in bicarbonate buffer, but no response to glucose unless a hydrogen acceptor, ferricyanide, is supplied. Young cells of unidentified strains of *Streptomyces* are reported by Präve (194) to carry out a fermentation under nitrogen. Experiments with partial anaerobiosis, e.g., those of Ganguly & Roy (77) are difficult to interpret.

Intact cells of *S. griseus* during aerobic respiration with glucose utilize inorganic phosphate and accumulate many of the well-known organic phosphates: glucose-6-phosphate, 3-phosphoglyceric acid, phosphopyruvate, and adenosine mono- and triphosphate; these determinations were made by Ganguly & Roy (78) using barium salt fractionation methods. Hockenhull *et al.* (118) report chromatographic detection of both glucose-1- and glucose-6-phosphate in fluoride-poisoned cells of the same species.

Respiratory mechanisms.—The appearance of relatively large amounts of lactic acid as a product of glucose metabolism by anaerobic or microaerophilic species of *Actinomyces*, as shown by Howell & Pine (121), suggests an Embden-Meyerhof pathway. Species of *Streptomyces* commonly form pyruvic acid aerobically as demonstrated, for example, by Sahay (205). Hockenhull *et al.* (118) showed that *S. griseus* forms lactic acid under conditions of restricted aeration; this, too, is a common observation.

Many of the known enzymes of the Embden-Meyerhof system have been detected in actinomycetes by conventional methods. Cochrane (37) described evidence for the presence in extracts of *S. coelicolor* of phosphofructokinase, aldolase, triose phosphate isomerase, phosphoglyceryl kinase, enolase, and ethanol dehydrogenase. It was suggested that the organism is nonfermentative because it lacks any anaerobic system able to regenerate reduced DPN. However, it should be noted that Nickerson & Mohan (177) found, in an acetone powder of *S. fradiae*, a DPN-linked lactic dehydrogenase.

Known enzymes of the phosphogluconate oxidation pathway have been detected in *S. olivaceus* by Maitra & Roy (158), in *S. aureofaciens* by Boretti and co-workers (17), and in *S. coelicolor* by Cochrane and associates [Cochrane & Hawley (39); Cochrane, Peck & Harrison (41)]. In *Nocardia* spp., Duff & Webley (65) found rather extensive accumulation of sedoheptulose and ribulose, characteristic products of the phosphogluconate pathway; however, enzyme studies of these organisms will be necessary before generalization is possible, since these products by themselves do not require the entire pathway for their formation from the substrates used.

Recent years have been marked by numerous efforts to use relatively simple isotope techniques to determine the balance of alternative respiratory pathways. Thus, Cochrane, Peck & Harrison (41), studying only the labeling of CO_2 from glucose-1- and glucose-6-C^{14}, suggested a role of the phosphogluconate pathway in *S. coelicolor*. Much more complete studies by

Wang *et al.* (256), on *S. griseus*, suggest, at most, a minor role for this pathway, the bulk of aerobic respiration following the Embden-Meyerhof pattern.

It seems, particularly from the work of Wood & Katz (139, 275) that the methods used in these studies pose grave difficulties of interpretation. At most, perhaps, data on glucose-carbon appearance in carbon dioxide can be used as the basis for other and more decisive investigations.

Di Marco (54) has suggested that the balance of the two major respiratory pathways is affected by the phosphate level. *S. aureofaciens* cells grown at high phosphate levels form more pyruvate than do those grown at low, and phosphate depresses the rate of conversion of ribose-5-phosphate to hexose and heptose phosphates. It is suggested, therefore, that the Embden-Meyerhof pathway is favored by phosphate and that the second phase of growth in this species, in which chlortetracycline is formed, is dominated by the phosphogluconate pathway.

In the biosynthesis of streptomycin, as studied by Silverman & Rieder (223), the rate of entrance of glucose carbons into L-glucosamine suggests that the phosphogluconate pathway is responsible for providing a major share of the intermediates in the transformation. Yagishita (277) reports the formation from glucose (but not from glycerol) of gluconic acid by *S. pyridomyceticus*, but the significance of this for respiratory pathways is not yet clear.

Evidence of a tricarboxylic acid cycle in *Streptomyces* spp. comes primarily from studies on crude extracts and from isotope distribution experiments; we will not cite the numerous determinations which indicate that acids are oxidized by whole cells or appear in cells or medium. Extracts of *S. coelicolor* were shown by Cochrane & Peck (40) to carry out several of the characteristic transformations of the cycle. More recently, Präve (194) has shown that extracts of *Streptomyces* sp. oxidize the known major acids of the cycle. Both Douglas & San Clemente (63) and Inoue (126) were able to demonstrate malonate inhibition of succinate oxidation, the former in intact cells, the latter in extracts.

Isotope distribution studies on *S. griseus* at Oregon State University have contributed more directly to the belief that the tricarboxylic acid cycle plays a role in actinomycetes. The incorporation of acetate into glutamate and the relative rates of appearance in carbon dioxide of the methyl and carboxyl carbons of acetate are consistent with the operation of the cycle [Gilmour *et al.* (86); Wang *et al.* (256)]. Butterworth, Gilmour & Wang (28) also showed that the incorporation of carbon dioxide into aspartate is consistent with a C_3+C_1 formation of 4-carbon dicarboxylic acids, and its incorporation into glutamate is consistent with the operation of the tricarboxylic acid cycle.

In addition to the work just cited, two other demonstrations of carbon dioxide fixation may be mentioned, viz., the work of Hirsch (110) on *N. petroleophila*, and that of Pine (190, 191) on *A. bovis*. In the latter study,

entrance of carbon dioxide into succinate seemed consistent with the operation of a C_3+C_1-fixation reaction. Such an enzymatic reaction has been shown in *N. corallina* by Baugh, Claus & Werkman (4):

$$\text{Phospho}\textit{enol}\text{pyruvate} + CO_2 \leftrightharpoons \text{oxalacetate} \quad 1.$$

There is strong evidence that inosine diphosphate is an obligatory participant in the reaction. Pine & Howell (193) found a CO_2 requirement for growth in several strains of *Actinomyces.*

Terminal respiration in the aerobic actinomycetes, specifically, *Streptomyces* spp., appears to be mediated by a more or less typical cytochrome system. In contrast to a report by Heim, Silver & Birk (107), more recent work of Neiderpruem & Hackett (178) indicates that in several *Streptomyces* spp. there are cytochromes of the three common types, *a*, *b*, and *c*. Spectroscopic determinations on *S. purpurascens* by Dölle (58) and by Sahay (205) indicate four components: *a*, a_3, *b*, and *c*. Inoue (126) also reported evidence indicating four cytochromes in *S. griseus*. A cytochrome of the *b* type has been partially purified from *S. fradiae* by Birk, Silver & Heim (14). Cyanide inhibition of respiration suggests that cytochrome systems are at least the major mechanism of terminal respiration (178, 205).

Several observations, none decisive, have led Nickerson & Mohan (177), Inoue (126), and Sahay (205) to suggest that some fraction of terminal respiration may be mediated by a flavoprotein rather than by a cytochrome system.

Lester & Crane (154) found no coenzyme Q (ubiquinone) in *S. griseus* and *Mycobacterium phlei*, but found both species to contain "large amounts" of vitamin K. This and other evidence suggests that the electron-transporting role of coenzyme Q may be taken in these organisms by one of the K vitamins. Jacobsen & Dam (128) report an unidentified member of the K group in *Nocardia* spp.

Catalase is, as expected, easily demonstrable in aerobic actinomycetes [Dölle (58); Giolitti (87); Kovács & Mathkovics (145)]. Members of the anaerobic genus *Actinomyces* lack catalase (142, 238).

Küster (149) found a phenoloxidase, assayed by oxidation of pyrogallol, in the mycelium of eight out of nine *Streptomyces* spp. studied. The phenoloxidase of *S. antibioticus*, according to Ševčík (219, 220), appears to be of the laccase type. Frommer (75) argues that the insensitivity of respiration to phenylthiourea shows that phenoloxidases are not involved in respiration.

Fatty acid metabolism.—Pine and co-workers (192, 193) have determined the fatty acids formed by *Actinomyces* spp. from glucose as propionate, acetate, and formate; in strains of *A. israeli*, the acid in highest concentration was propionate. This recalls the possible relationship to *Propionibacterium;* studies on the metabolism of propionate in *N. corallina* by Martin & Batt (167) indicate two pathways of propionate formation, one via succinate, the other not. Earlier, Hungate (125) had shown propionic acid to be the principal fermentation product of an anaerobic *Micromonospora*, *M. propionici.*

Acetate is at least a minor product of most *Streptomyces* spp.; it was reported by Perlman & O'Brien (185) to be a major product of *S. fradiae* in an unbuffered glucose medium. Präve (194) identified acetate, butyrate, valerate, and propionate by paper chromatography of the culture medium of unidentified *Streptomyces* isolates.

Midwinter & Batt (171) have made an important study of acetate metabolism in *Nocardia corallina*. Oxygen uptake with acetate as the substrate amounts only to 50 per cent of the theoretical value, with a respiratory quotient of unity; this suggests, of course, an oxidative assimilation similar to that known for some years in bacteria. Oxidative assimilation is largely abolished by azide or 2,4-dinitrophenol. The primary assimilate from both acetate and propionate in this organism is presumably a carbohydrate; the specific activity of carbohydrate in cells incubated with propionate-3-C_{14} is much higher than that of cellular lipids. With *N. corallina*, earlier work of Martin & Batt (167) had shown that propionate is metabolized via pyruvate and that carbon dioxide stimulates the conversion.

Studies made by Hirsch & Wallace (114) on octanoate oxidation by *S. aureofaciens* suggested the β-oxidation pathway, as in other organisms.[3] Webley, Duff & Farmer (263, 265, 266) have investigated β-oxidation in *Nocardia* spp., primarily, *N. opaca*. Among the ω-substituted fatty acids, those with an even-numbered acid side chain—phenylacetic, phenylbutyric, and phenylcaproic acids—are oxidized by resting cells to *o*-hydroxyphenylacetic acid, but phenyl-substituted acids with odd numbers of carbon atoms (phenylpropionic, etc.) are oxidized to different products (263). In the conversion of γ-substituted chlorophenoxybutyric acids, the expected β-hydroxy derivative of the original substrate has been identified (265). Finally, study of the breakdown of alkylcarboxylic acids confirms that the β-hydroxy derivative is formed, its accumulation in the medium being affected by the position of chlorine introduced into the aromatic ring (266).

Steroid transformation.—The practical importance of the steroids has been the stimulus for a large volume of work in this field. Steroid transformations by actinomycetes and other microorganisms have been reviewed in detail by Eppstein *et al.* (67), Stoudt (236), and by Wettstein (271), and these reviews will not be duplicated here.

Sih & Bennett (222) isolated a cell-free system from *Nocardia* sp. able to dehydrogenate steroids having the 5β- or 5α-pregnane nucleus; the natural hydrogen acceptor is not known, and presumably at least three enzymes must be invoked to account for the reactions displayed.

Lindner and co-workers (156) in 1958 described *Streptomyces hydrogenans* as characterized by the ability to reduce 20-keto steroids to the corresponding 20β-hydroxy compound. The reaction is stereospecific: the 20α-hydroxy analogue never appears. The enzyme, 20 β-hydroxy-steroid dehydrogenase,

[3] This paper was cited incorrectly by Cochrane (38) as having provided evidence against β-oxidation.

has been purified and crystallized by the same group of workers (122, 123, 124). Reduced DPN is 10 times as active as reduced TPN in the system. The enzyme is formed in small amounts in mycelium grown in the absence of steroid, but is increased 10- to 20-fold by the addition to the culture medium of a variety of C_{21} steroids (176).

Most of the steroid transformations so far studied have been relatively limited, hydroxylation, reduction, etc., at one or two positions. It is known, however, that actinomycetes can utilize steroids as a source of carbon with, presumably, extensive or perhaps complete degradation. Hayakawa and coworkers (103, 104, 105) have undertaken a study of the breakdown of cholic acid and related compounds by *S. gelaticus* and *S. rubescens*. Some of the early breakdown products have been identified and arranged in a possible metabolic sequence on the basis of their structure. Halperin, Quastel & Scholefield (101) report a DPN-linked cholic dehydrogenase from a soil actinomycete; cholic acid is converted to an unidentified derivative of 3-ketocholanic acid.

Other aspects of carbon metabolism—Cain (29) showed that the oxidation of *p*-nitrobenzoic acid by *Nocardia erythropolis* is adaptive, as is the oxidation of the *ortho* isomer by *N. opaca;* oxidation of these two isomers by competent organisms is completely inhibited by the *meta* isomer. Cain & Cartwright (30, 31, 32) have made considerable progress in elucidating the path of carbon in the breakdown of the nitrobenzoic acids by *Nocardia* spp. Cells of two isolates form protocatechuic acid oxidase only if grown on nitrobenzoate, although in *N. opaca* the enzyme is present in glucose-grown cells. On the basis of several types of evidence these authors propose the following pathway for oxidation of *m*- and *p*-nitrobenzoic acids:

$$\text{Nitrobenzoic acid} \rightarrow \text{protocatechuic acid} \rightarrow \beta\text{-carboxymuconic acid} \\ \rightarrow \beta\text{-ketoadipic acid} \rightarrow \text{succinic acid.} \qquad 2.$$

The enzyme responsible for the third reaction above was separated from homogenates. The system as a whole is similar to that known in *Neurospora* and in *Pseudomonas,* although there are probably differences in detail.

Nocardia opaca forms a catechol oxidase, converting catechol to *cis-cis*-muconic acid (30). Wieland, Griss & Haccius (273) detected *trans-trans*-muconic acid as a product of benzene oxidation by *N. corallina;* the authors suggest that catechol, but not phenol, is an intermediate.

From a careful analysis of the breakdown of aliphatic hydrocarbons, Webley, Duff & Farmer (264) present evidence that hydrocarbons are converted to fatty acids, by oxidation, and that further metabolism follows the β-oxidation pathway already described.

Hirsch & Alexander (112) studied the metabolism of halogenated fatty acids by isolates of *Nocardia*. 2,2-Dichloropropionic acid undergoes both dehalogenation and oxidation of the carbon chain; insertion of halogen into the 3-position of propionate prevents its decomposition.

Acetoin appears as a minor metabolic product in studies made by Davis & Freer (53) on *N. salivae*, and by Pine & Howell (193) on *Actinomyces* spp.

Musílek and others (173) demonstrate its formation by *S. erythreus;* acetoin formation is increased by phosphate in high concentrations and by arsenite poisoning. Biosynthesis from pyruvate and acetaldehyde is suggested (172).

Oxalic acid formation is reported by Präve (194), recalling its earlier demonstration by Perlman (182) in *Streptomyces* spp.

Nitrogen Nutrition and Metabolism

Nitrogen fixation.—The long-standing controversy over nitrogen fixation in actinomycetes is reviewed by Waksman (252). Metcalfe & Brown (169) demonstrated quite active fixation, comparable in rate to that of *Azotobacter*, in *N. cellulans*. Fixation by *N. calcarea* was somewhat slower but definite. Fedorov and co-workers (69, 71) surveyed a large number of strains and detected fixation, never at high levels, in about 10 per cent of the cultures. Quispel (197) reviews the problem of the endophytic mycorrhizal organism of *Alnus glutinosa*, but it cannot yet be said with certainty that this organism is an actinomycete.

Nitrification.—Schatz and his associates (127, 210, 211) isolated *Streptomyces nitrificans*, an organism which oxidizes urethane with formation of nitrite. Other carbamates, ammonia, and urea are also oxidized to nitrite. A role of carbamyl derivatives as intermediates is unlikely, since N-substituted carbamates are not oxidized by urethane-adapted cells.

Jensen (133) found that *N. corallina* oxidizes pyruvic oxime to nitrite; in a peptone medium 90 per cent of the oxime nitrogen was recovered as nitrite. Later work of Lees *et al.* (152) showed the same organism to be able to oxidize hydroxylamine to nitrite. Neither in this species nor in *S. nitrificans* is nitrate formed, and there is no evidence that the oxidations described provide useful energy to the organism.

Nitrate metabolism.—Nitrate reduction is common but not universal in the actinomycetes; Waksman (252) reviews the literature on nitrate as the nitrogen source. Not all actinomycetes can utilize nitrate; however, separation of the ability to reduce nitrate from the capacity to grow on it as sole source of nitrogen has not always been achieved. It has been found, e.g., by Mariat (163) that nitrite is generally toxic in an appreciable concentration.

Fedorov & Kudryashova (70) briefly report slow anaerobic growth of *Actinomyces* (*Streptomyces*) spp. in the presence of nitrate, and the formation from nitrate of gaseous nitrogen. However, the claim for denitrification rests only on failure to recover all of the nitrogen supplied; furthermore, the amount of growth reported is extraordinarily low.

Utilization of organic nitrogen.—Utilization of urea is uncertain; although Gottlieb (91) finds urea a suitable nitrogen source for only one species. Stapp (234) and Stapp & Spicher (235) find growth on urea, as estimated, however, by visual means only, common. Mariat (163), by similar methods, rated urea as one of the more utilizable nitrogen sources for *N. asteroides* and *S. pelletieri*. Part of the difficulty may arise from ignorance of the fact that urea breaks down to ammonia on autoclaving; hence only results with cold-

sterilized urea are valid. Simon (224) detected urease in the mycelium of *S. griseus*, possibly related to the presence in this species of deguanidases (see below).

Amino acids as nitrogen sources are surveyed by Kurz & Nielsen (148), with *S. griseus*. Both Tendler (243) and Webley (266) report specific amino acid requirements for thermophilic species. Other utilizable nitrogen sources reported in recent years include cyanide by Ware & Painter (259), humic acids by Schönwälder (215), and uracil by Martin & Batt (166). As yet unspecified organic nitrogen requirements are demonstrated by Mariat (161).

Keratin has long been thought to be attacked by actinomycetes, and Noval & Nickerson (181) provide for the first time unequivocal proof that highly purified keratin is, in fact, broken down by a number of species of *Streptomyces*. Piechowska (189) reports also on keratin hydrolysis by *Streptomyces* spp.

Proteolytic enzymes.—Recent Japanese work on a protease of *S. griseus* is reviewed fully by Hagihara (99). The enzyme has been crystallized. Its outstanding characteristic is a wide range of activity; Nomoto, Narahashi & Murakami (179) report that, not only are a variety of proteins attacked, but that a given protein, casein, is much more extensively hydrolyzed than it is by such well-known proteases as trypsin. The enzyme is stabilized by calcium and irreversibly inactivated by ethylenediaminetetraacetic acid (179). Tytell *et al.* (248) report a protease, resembling but not identical with this one, which may require zinc for activation.

Amino acid metabolism.—Gottlieb & Ciferri (92) report on rather extensive deaminase activity in *S. venezuelae;* in general only those amino acids which are deaminated can support growth, suggesting that at least a part of the cellular nitrogen enters into metabolism as ammonia. Transaminase activity, however, is suggested by several data: those of Crawford (48) on *N. globerula*, of Präve (194) on *Streptomyces* sp., of Douglas & San Clemente (64) on *S. scabies*, and of Romano & Nickerson (202) on *S. fradiae*.

Arginine metabolism in *S. griseus* is of interest because of its possible relation to streptomycin synthesis; the transamidase of Walker (255) could function in biosynthesis. Roche and his co-workers (247) described deguanidases which split off urea from arginine and other mono-substituted guanidines, including streptomycin; presumably, this system operates in streptomycin destruction by the cell. Cells of *S. griseus* also oxidize arginine to guanidinobutyramide (245, 246); the reaction is an oxidative decarboxylation and can be effected only aerobically. Thoai & An (244) describe a specific amidase acting on guanidinobutyramide.

Lysine synthesis, reviewed by Vogel (250) and by Vogel & Bonner (251), groups the actinomycetes with the Eubacteriales—lysine is synthesized by way of diaminopimelic acid. The higher fungi use another pathway, although certain of the *Saprolegniales* synthesize lysine as do the actinomycetes (250). This again encourages us to classify actinomycetes with the true bacteria but to recognize the possibility of affinities, perhaps through *Actinoplanes*

and *Streptosporangium* of Couch (46, 47), with the lower Phycomycetes.

Cain (29) found that resting cells of *N. erythropolis* form aspartate if provided with fumarate and ammonium ion, suggesting the presence of an aspartase.

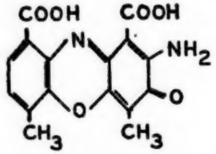
Other aspects of nitrogen metabolism.—The metabolism of the carbon of nitrobenzenes has been mentioned earlier. Cain & Cartwright (31, 32) have detected aminophenyl compounds formed by *N. erythropolis* from *p*-nitrobenzene, but it is not certain that the expected *p*-aminobenzoic acid is an intermediate. Another species of *Nocardia* accumulates nitrite from *m*-nitrobenzoic acid.

Koaze (143) has determined the formation by *Streptomyces* sp. of two synergistically acting, seed germination-promoting substances, L-prolyl-L-valine anhydride and 1-methyl-2,4-imidazoledione. Synthesis of a number of amides has also been reported. Sekizawa (218) identified *trans*-cinnamic acid amide and ethoxyethene-1,2-dicarboamide in culture filtrates of *Streptomyces* sp. grown on a complex medium, and workers in two laboratories (239, 242) have isolated acetylenedicarboxamide as a metabolic product of *Streptomyces* spp. N-acetyltyramine is formed by a strain of *S. griseus* (42). Jeffries, Holtman & Guse (132) demonstrated nucleases in *S. rimosus* and *S. albus* by an agar plate method.

Biosynthesis

The search for new antibiotics continues to uncover new chemical species, and it is obvious that fascinating problems of biosynthesis are posed. Other reviews, e.g., those of Binkley (13), Burkholder (27), and Waksman & Lechevalier (254), should be consulted for the antibiotics; here we shall be concerned only with actual investigations of biosynthetic mechanisms.

Actinomycins.—Actinomycins, produced by various species of *Streptomyces*, are heteromeric peptides and contain a chromophore designated as actinocin by Brockmann (25):



3.

Peptide chains of five amino acids are attached to each of the carboxyl groups by amide linkages. In all actinomycins threonine residues are attached directly to the chromophore.

Brockmann *et al.* (25, 26) suggested from chemical evidence that actinomycins might be formed in nature by the oxidative condensation of two peptides attached to 3-hydroxy-4-methylanthranilic acid. Tryptophan is there-

fore of some interest as a possible precursor. Sivak & Katz (227) briefly report that tryptophan is converted by *S. antibioticus* to 3-hydroxyanthranilic acid. Katz (135) finds carbon 14 from the 7-position of tryptophan incorporated into the chromophore of actinomycin. These initial findings point to a possible synthetic pathway from tryptophan, via kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and (speculatively) 3-hydroxy-4-methylanthranilic acid. A conversion of tryptophan to anthranilic acid by other species of *Streptomyces* is suggested by Kawamata, Koyama & Kunita (140). The role of tryptophan is further indicated by the observation (135) that analogues of it inhibit actinomycin synthesis and that the inhibition is reversed by tryptophan or kynurenine.

Katz (135) and Schmidt-Kastner (214) summarize the effect of amino acids on the type of actinomycin produced. To take one example from the work of Katz & Goss (90, 138), the addition of sarcosine to cultures of *S. antibioticus* decreases the relative amount of actinomycin "IV" and increases the amounts of types "II" and "III," both of which have more sarcosine units. Comparable shifts are caused by supplementation with hydroxyproline (137).

Apparently non-specific effects, e.g., the change in actinomycin components observed by both Frommer (76) and Martin & Pampus (165) with changes in the carbon:nitrogen ratio of the medium, can be presumed to reflect changes in the synthesis by the organism of the constituent amino acids of actinomycin.

The addition of precursors induces the formation of new actinomycins. Schmidt-Kastner (213, 214) describes the formation of "E" and "F" actinomycins by *Streptomyces* sp. in media supplemented with amino acids. Some of these may be identical with those of Katz & Goss (138). Katz (135) reports the formation of new actinomycins when L-pipecolic acid is supplied to *S. antibioticus*.

Studies by Katz (136) show that, although the predominant actinomycin of the strain used contains D-valine, supplementation of the medium with this isomer actually reduces total synthesis. Since the effect of the D-valine is reversed by L-valine, it is concluded that the D-valine of the actinomycin is synthesized from the natural isomer, although no evidence is as yet at hand for the mechanics of the transformation. MacDonald (157) finds a similar situation in valinomycin biosynthesis.

Systems of classification of the actinomycins and of the organisms producing them are discussed by workers from the principal interested laboratories: Bossi *et al.* (20), Brockmann (25), Waksman, Katz & Vining (253), and Woodruff & Waksman (276).

Streptomycin.—Studies of streptomycin synthesis to 1959 are reviewed in detail by Hockenhull (116). Silverman & Rieder (223) isolated N-methyl-L-glucosamine from streptomycin formed with D-glucose-1- and glucose-6-C^{14}. The distribution of label excludes a biosynthesis based on L-glucose or a cyclic

hexitol as intermediates; a possible explanation involves operation of the phosphogluconate oxidation pathway plus epimerizations, but no final mechanism can be described.

Chlorine-containing antibiotics.—Cultural conditions for the formation of tetracyclines are reviewed by Di Marco & Pennella (56). Chlortetracycline biosynthesis affords some insight into biological chlorination. Wang (257) showed that the chlorination step probably precedes final synthesis of the carbon skeleton, since supplied tetracycline is not chlorinated; one of three unidentified organic chlorine products supplies the chlorine for antibiotic biosynthesis.

Inhibition of chlortetracycline biosynthesis by bromide is described by Sekizawa (217), Gourevitch and others (94) and Doerschuk *et al.* (57). Not all strains react alike, but often the effect of bromide is the replacement of chlortetracycline by tetracycline and, at high bromide concentration, bromtetracycline. Presumably, bromide competes with chloride for a position on some unknown precursor. Other inhibitors of chlortetracycline formation have been studied by Lein, Sawmiller & Cheney (153) and by Goodman *et al.* (89), but no clearly defined explanation of their action can now be put forward.

Wang and his associates (258) suggest, on the basis of studies with chlorine-36, that dichloroacetic acid may be a precursor of chloramphenicol. The possibility that *p*-nitrophenylserinol is a chloramphenicol precursor seems to be eliminated by the work of Gottlieb, Robbins, & Carter (93).

Erythromycin.—Two preliminary reports (43, 95) indicate that the erythronolide chain of erythromycin is built of propionate units, probably incorporated without randomization. Propionate does not enter into the sugar moieties of the antibiotic.

Vitamin Requirements and Vitamin Synthesis

Vitamin requirements.—Since requirements for the common vitamins (and other growth factors) appear in radiation-induced mutants of actinomycetes, e.g., in the work of Bradley & Lederberg (22) and Saito & Ikeda (206), it is to be assumed that in their general requirements those organisms are not distinguishable as a group from others. Specific requirements uncovered during the period covered by this review include: that of *Nocardia* spp. for the pyrimidine moiety of thiamine, reported by Mariat (161, 162); thiamine, riboflavin, and biotin requirements in a thermophilic species shown by Tendler (243); and the requirement of *M. vulgaris* for biotin (partially replacable by Tween 80) reported by Webley (261). Martin & Batt (166, 167) found that the requirement of *N. corallina* for thiamine is not apparent if certain amino acids are provided, and suggest that thiamine is required for the incorporation of ammonium ion into amino acids.

Riboflavin.—A fluorescent pigment active in riboflavin bioassay but not identifiable as riboflavin or either of the flavin nucleotides, was discovered in

Streptomyces sp. by Ciferri & Machado (34); it is pertinent to recall the claims made by Hata *et al.* (102) and by Protiva (196) of riboflavin formation in actinomycetes.

The vitamins B_{12}.—The synthesis of the B_{12} vitamins has received much more attention. Darken (51) reviewed their occurrence and production by microorganisms, including actinomycetes. The review by Johnson & Todd (134) emphasizes the chemistry of the compounds, and Perlman (184) summarizes our knowledge of microbial biosynthesis. Basic physiological data on cultural requirements for maximal production are presented by Hall and co-workers (100). Ledingham (151) has reviewed industrial production, Coates & Porter (36), the role of B_{12} vitamins.

So far as is known, the B_{12} vitamins are microbial products only; surveys of actinomycete populations by Janicki *et al.* (129), Letunova (155), and Witkùs *et al.* (274) indicate that the capacity to form B_{12} is widespread in the group.

Makarevitch, Verkhovtseva & Laznikova (160), working with *Actinomyces* (*Streptomyces*) *olivaceus*, found that aeration improves the yield of vitamin B_{12} and that at low aeration pseudovitamin B_{12} (adenylcobamide) is formed in a substantial amount. Surikova & Popova (237) report improved yields of B_{12} with cobalt supplementation of a complex medium; the vitamin is formed in the mycelium, as observed earlier in other actinomycetes (184). Perlman & O'Brien (186) found cobalt-accumulating yeast cells to be a better source of cobalt than were inorganic salts.

Mention has been made of pseudovitamin B_{12} as an actinomycete product. Other analogues may be obtained from *Streptomyces* spp. by the provision of specific precursors. Fantes & O'Callaghan (68) induced the formation by *S. griseus* of the α-benzimidazolyl analogue by providing *o*-phenylenediamine. The same authors and others have extended this approach; the literature to 1958 is summarized by Perlman (184). Boretti and associates (16) report on analogues formed by *N. rugosa* under the influence of other phenylenediamines.

Work on actinomycetes is, of course, only part of the continuing study of the biosynthesis of the B_{12} series. *N. rugosa* in culture forms a guanosine diphosphate derivative of factor B (the cobalt porphyrin nucleus of B_{12}); Barchielli and co-workers (2) suggest that this compound may, by analogy with known reactions, exchange with a riboside to form vitamin B_{12}. The guanosine diphosphate is partially active for *Escherichia coli* (79). *N. rugosa* is reported by Di Marco and his co-workers (3, 55) to form other presumed B_{12} precursors: uroporphyrin, coproporphyrin, and a monophosphate of Factor B. Bernhauer, Becher & Wilharm (8) describe the conversion by *Streptomyces* sp. of Factor A and Factor III to vitamin B_{12}. Muto *et al.* (174) briefly report the formation by an enzyme preparation from *S. olivaceus* of vitamin B_{12} from a number of the known structural components.

Labeling studies with actinomycetes have also contributed to our

knowledge of biosynthesis. Shemin and co-workers (23, 44, 221) have shown that: (*a*) δ-aminolevulinic acid is a precursor of vitamin B_{12} as it is of other porphyrins; and (*b*) methyl groups of the porphyrin which would not be expected to come from δ-aminolevulinic acid are supplied by methionine. Krasna, Rosenblum & Sprinson (146) suggest that the aminopropanol residue attached to the porphyrin of vitamin B_{12} arises from L-threonine. Weygand, Klebe & Trebst (272) had earlier shown that labeled 5,6-dimethylbenzimidazole is incorporated into the B_{12} molecule.

Iron compounds.—A new type of growth factor containing iron has been under study intensively in recent years. The best known are coprogen, the ferrichromes, and the *Arthrobacter terregens* factor; these are reviewed by Neilands (175). Of these three, only coprogen is so far known, from the studies of Hesseltine and associates (109), to be produced by actinomycetes. All of these are similar in that they are iron compounds and are growth factors for certain common microorganisms; they are by no means identical, although Neilands (175) has suggested tentatively that all function in iron transport. There is, however, no obvious relation to the iron compound ferroverdin, isolated by Chain, Tonolo and Carelli (33) from *Streptomyces* sp. Ehrenberg (66) finds, for example, that ferrichrome and ferrichrome A contain ferric iron, while ferroverdin contains ferrous iron.

Recent studies of Swiss workers provide a more general conceptual framework for these and other iron compounds of actinomycetes. The ferrioxamines have been defined by Zähner, Hütter & Bachmann (278) as being of general occurrence and probably essential for all cells; no structure can as yet be given, but ferrioxamine B, from *S. pilosus*, contains iron and chlorine and yields on hydrolysis 1-amino-5-hydroxylaminopentane (10). Bickel and others (11) conclude tentatively that a group of antibiotics, grisein, albomycin and the ferrimycins, may be classed together as sideromycins. Coprogen, ferrioxamines, and the ferrichromes are then defined as sideramines and can be characterized by their ability to reduce toxicity of the sideromycins as well as by their growth factor activity for various organisms and by their iron content. Undoubtedly, before this review is printed, more will be known of the fascinating iron metabolites.

Mineral Nutrition

Webley (262) has reported careful and quantitative experiments showing the requirements of *Nocardia opaca* for manganese, iron, and zinc, and has described several deficiency symptoms. Visual estimates of growth of *Streptomyces* sp. show, according to Spicher (233), that the promotion of growth by soil extract is a mineral effect, and that iron, manganese, copper, zinc, and molybdenum increase growth. Maitra & Roy (159), using dry weight as the criterion of growth, find positive effects on *S. olivaceus* of the just-mentioned four elements and of cobalt and chromium; magnesium is reported fully replaceable by manganese. Heim & Lechevalier (106) confirm

iron and zinc requirements for *Streptomyces* spp. and Nickerson & Mohan (177) show dry weight increase in *S. fradiae* with added calcium. Khaloupka (141) has demonstrated the requirement of *S. griseus* for potassium.

Iron toxicity to *S. rimosus* has been studied by Horvâth and associates (119, 120) but without growth data; inhibition of antibiotic production by iron in media containing unsaturated fats is credited to peroxide formation.

Lytic Factors

Ghuysen (82), and earlier, Welsch (267), provide reviews of the long and somewhat confused history of the lytic action of actinomycete products on other bacteria.

Salton (207, 208) divides lytic action into two fundamentally different types, although a given *Streptomyces* sp. may exhibit both. The first, lysis of heat-killed bacteria, is almost certainly to be attributed to proteolytic enzymes, as was shown first by Ghuysen (80, 82, 84). Whether there is only one or, as is more likely, several of these proteases is not entirely clear.

The enzyme mixture from *S. albus* which lyses living cells or separated cell walls of staphylococci and some other Gram-positive bacteria, was determined by Ghuysen (82) to contain two separable components, F_1 and F_2. These act alone, although bacteria differ in their sensitivity, and they act synergistically when both are present. The most recent work of Ghuysen (83) and Ghuysen & Salton (85) indicates that F_1 resembles lysozyme in having N-acetylhexosaminidase activity, but differs from it in several important respects. The preparation is heterogeneous and contains one or more peptidases in addition to polysaccharidase activity. Welsch (269) found this preparation to be active against several *Streptomyces* spp.

A purified fraction, F_2B, appears to be, from the work of Ghuysen (83), an amidase acting on the muramic acid-peptide linkages of the cell wall.

Distinct again from this complex, although also formed by *S. albus*, is an enzyme system acting on cell walls of streptococci but not lysing *Micrococcus lysodeikticus*. This system is found by Salton (207) in species of *Streptomyces*, *Nocardia*, and *Micromonospora*. Slade & Slamp (230) find that this preparation is active against the cell membrane as well as against the cell wall of *Streptococcus pyogenes;* rhamnose and hexosamine are liberated from the cell wall.

A fourth class of lytic substances act on pneumococci; Ghuysen (81) briefly describes action on pneumococci as attributable to a complex of four factors, two of them specific.

Still other lytic activities have been described but not yet studied in detail. Welsch, Ghuysen & Castermans (270) report in *S. albus* a system, distinct from those so far described, that is able to lyse cells of Gram-negative bacteria at 60 to 65°C. but not identical with that lysing heat-killed Gram-negative bacteria. Skinner (229) reported that *S. alboflavidus* produces a substance able to lyse the mycelium of a fungus, *Fusarium culmorum*.

Thus, an apparently single "substance," Welsch's actinomycetin, is seen to be extremely complex; not only are many different lytic factors and proteases present but different species can be expected to, and do, form them in different amounts, as has been shown by Bergamini, Ghuysen & Welsch (6) and by Welsch (268).

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CONTINUOUS CULTURE OF MICROORGANISMS[1,2]

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It is perhaps highly appropriate that a reviewer of a specific area of scientific literature be chosen from the ranks of investigators who have not as yet contributed to the area being reviewed. This is not to say that the subject of continuous culture of microorganisms is not of great interest to this reviewer but rather that it permits him a degree of freedom not open to those involved in the subject. The review will reflect the bias of the reviewer since it will emphasize the cell rather than the culture as the subject of discussion.

On first contact with the literature one gains the impression that "the name's the thing," because many investigators, perhaps with tongue in cheek, have christened their inventions with such labels as Chemostat, Bactogen, Turbidostat, Breeder, Auxanometer, Microgenerator, and Symbiocon (1). Whatever the genesis of these colorful names, they mark the individuality of the inventors and assure the reviewer that to overlook a paper by one's colleague is not too grievous an error.

A candid review should start with recognition of the most important contributions to the field which are represented by the early work of Myers (2), the insights of Monod (3), and Novick & Szilard (4). As a review was written on this subject in 1955 (5) and a symposium published in 1958 (6), this topic will be developed with some repetition. We feel the material can stand re-emphasis owing to its many ramifications.

Since one might ask, what is a continuous culture and what is a microorganism, definitions are warranted before becoming too involved with names. To answer the last question first, we will arbitrarily define microorganism to include any cell type that can be grown in suspension, namely, bacteria, algae, protozoa, tissue culture cells, etc. Fortunately or unfortunately, we must exclude fungi and the like for want of space. The phrase "continuous culture" is self-explanatory, but tautologically we will consider it to be a culture of cells of any type into which there is a continuous addition of fresh medium for the purpose of maintaining the culture at some steady-state level of growth. We will not include industrial applications of these techniques.

Externally and Internally Controlled Continuous Cultures

Novick distinguishes two types of continuous culture systems in his review of bacterial growth (5). He suggests that differentiation be based on

[1] The survey of the literature pertaining to this review was concluded in December, 1960.

[2] The following abbreviations will be used: DNA (deoxyribonucleic acid); RNA (ribonucleic acid).

the method of control. First, the external type are those systems externally controlled by the use of a fixed flow rate such that the flow does not equal or exceed the maximum growth rate of the microorganisms being cultured in the presence of a limiting nutrilite. Such systems are controlled externally by the operator. Second, the internally controlled systems are those that rely on a sensing element such as a photocell to detect some internal change in the culture, i.e., optical density of the cells, to regulate the input of fresh medium with an increase or decrease of cell population. This design operates at a flow rate which may match the maximum growth rate.

To make this distinction meaningful it will be helpful to summarize the basic formulation presented by Monod (3) and also by Novick & Szilard (4). We will use the notation of Novick (5) which applies to his Chemostat as well as to Monod's Bactogen. It can be modified to apply to internally controlled systems as well. The key to the success of this method of continuous culture operation was the recognition that an equilibrium between population growth and loss by overflow could be obtained through the use of a fixed input flow rate of the culture medium. This is done in the presence of a single growth-limiting factor with all other factors in excess. The relationship is formulated by considering logarithmic growth in a batch culture. It is expressed as a differential which states that the change in cell number, N, per ml. with time, t, i.e., dN/dt is proportional to the number of cells or equal to the number of cells times a constant α, written as

$$dN/dt = \alpha N \tag{1.}$$

On integration, this leads to the well-known statement for logarithmic growth. Viability is assumed to be 100 per cent.

$$N = N_0 e^{\alpha t} \tag{2.}$$

By such a derivation, α is the growth rate constant and is related to the time required to double the number by the following equation:

$$\alpha = ln2/\tau \tag{3.}$$

The doubling time and mean generation time, τ, are generally assumed to be the same. Doubling time refers to doubling in mass; while the mean generation time refers to doubling in number. The latter should be qualified by referring to it as the "mean effective generation time," $\bar{\tau}$. Since the individual generation times of organisms in the culture are not all the same, one is obliged to recognize that it is the mean of these that obtains in any determination of this function. Confusion may enter the picture if these constants are calculated from raw data in which logarithms to base 10 or 2 of the numbers or amounts are used. *Alpha* is the slope of a curve using natural logarithms of numbers or mass, and the generation time is as stated above $ln\ 2/\alpha$ or $.693/\alpha$. If, however, logarithms to the base 10 are used, the slope of the curve, β, will be related to the generation time by $\tau = .301/\beta$. Finally, if $\log_2$ is used in the calculation, the slope, γ, is related to the generation time by

$\tau = 1/\gamma$. In this instance, γ is the reciprocal of τ. There are some workers who define the generation time as the reciprocal of α (7) and the doubling time by the formula $\tau = ln\ 2/\alpha$. This does not seem consistent with the accepted meaning of the terms (8), although it should be recognized that the definition of a term is the prerogative of the investigator.

In a continuous culture vessel of volume, V ml., into which medium from a sterile reservoir is allowed to enter and leave by overflow at a rate of w ml./unit time, w/V is the fraction of the culture lost per unit time and there will be w/V times the number of cells per milliliter lost in that time. This washout will decrease the number of cells in the culture vessel, and therefore the change in number with respect to time will be the increase by growth, αN, minus the loss by washout, $w/V \cdot N$, or expressed as an equation it can be stated as:

$$dN/dt = \alpha N - w/V \cdot N \qquad 4.$$

or

$$dN/dt = N(\alpha - w/V) \qquad 5.$$

When the loss of cells by washout $w/V \cdot N$ is greater than the increase of cells by growth, αN, the rate of change of cells in the vessel will be negative and the number will decrease eventually to zero. When the increase in cells, αN, is greater than $w/V \cdot N$, there will be an increase of cell number in the culture vessel. In this formulation for the Chemostat it was recognized that in the second case the increase would continue until the cellular utilization of the limiting nutrient or growth factor in the medium became determinate to the growth rate of the cells. When the growth factor concentration becomes limiting, the cells respond by a decrease in their rate of growth with the result that a succession of new growth constants appear until the equilibrium growth constant α' is established such that $N\alpha'$ equals $w/V \cdot N$. The rate of increase of cell number with time thus becomes zero and a constant number of cells in the growth flask ensues.

Equations 4 and 5 are based on 100 per cent viability of the cells. If this assumption does not hold, another expression for the loss of cells by death must be introduced (9). The distinction between the internally and externally controlled continuous culture can now be made. As previously stated, in the latter system a photocell or other detector senses the population density which, through relays or other electronic devices, increases the flow rate when the culture density increases, or it decreases the flow rate when the density falls because of washout. In terms of equation 4, α is maximal and remains so, while w/V increases with population density until it is equal to α. At equilibrium, the growth rate, α, is maximal for the medium and the flow rate rises to match it. It will operate in this region of maximal logarithmic growth provided the population density at which w/V becomes equal to α does not exceed the nutritive capacity of the medium and cause a decrease in α.

The distinction between the two methods is one based on their operation

in terms of the range of growth rates which can be studied. The external system cannot operate at maximum growth rate since slight momentary changes in growth rate can lead to a complete washout of the cells. How well an internal system would operate in the low growth rate region is dependent upon the design since changes in cell number at slow growth rates requires sensing elements of high sensitivity. The tendency for microorganisms to attach to light ports, photocells, or other sensing devices in the internally controlled system is the source of greatest technical difficulty even when operated at maximum rate, since this gives rise to a drift in the system caused by an accumulation of cells on these surfaces as the age of the culture increases. The problem of coating has been solved by several investigators (10 to 14). The stability of the internal system is a function of the machinery used in its construction; while the stability of the steady-state in an external system is a function of cellular regulation and is inherently better. This stability has been demonstrated mathematically by Spicer (15) both for the utilization of growth factors and for the production of growth inhibiting substances. Powell (8) asserts that there is no essential difference between the two systems and points out that with the Chemostat or Bactogen the experimenter fixes the flow rate, and therefore the growth rate, while the population level adjusts to the input nutrilite concentration. In the internally controlled system (2, 10), the experimenter selects the population density and the flow rate adjusts itself to the growth rate as it, in turn, is a function of the medium. The only question an investigator need decide is which method will offer the best control for the region he wishes to investigate.

Concentration-Dependent Growth Rate

The dependence of growth and population density on the concentration of the limiting growth substances in continuous cultures is an important aspect of these systems. Initially, Monod (16) clarified the relationships through his analysis of growth rates as a function of concentration of nutrilites. Although the first work on concentration dependence of the growth rate was done in batch cultures, the utilization of a continuous culture apparatus greatly improves and simplifies the procedure. If the steady-state concentration of a limiting growth factor or nutrilite present in the growth tube is determined at a series of growth rates (washout rates), it will be found to fit a hyperbolic function similar to the Michaelis-Menton equation for enzyme substrate interaction. This relationship is not strictly fortuitous since adsorption, transport, and the enzymatic utilization of an agent essential to growth fit into this general category of reactions. A plot of growth rate constants against the concentration of a single limiting growth factor in the growth tube is comparable to a plot of enzyme rates against substrate concentrations.

The relationship derived by Monod (3) states that the growth rate, μ, at a particular concentration, s, is equal to the maximum growth rate, μ_m, times

the ratio of the substrate concentration over the sum of the substrate concentration plus the concentration that gives one-half the maximum growth rate K_s.

$$\mu = \mu_m s/(s + K_s) \qquad 6.$$

This growth rate expression has been incorporated into the basic equation 4 for operation of a continuous culture in a steady-state (3). It gives the complete relationship between the rate of change of cell numbers, flow rate, and concentration of the limiting growth factor.

The data on the growth of a variety of microorganisms seem to fit equation 6 rather well. Both *Escherichia coli* and *Mycobacterium tuberculosis* behave in this way with respect to concentration of various sugars (16, 18). *E. coli*, strains B/1 t and B/1 tf (4), and several other strains (19) follow the same pattern as a function of the concentration of limiting factors. The precision with which these data fit such a function is considered by Moser in his monograph (9). He questions the equation on the basis that the over-all velocity of such a system does not result from a single "master reaction." He presents a general equation which retains the above symbols and is derived by multiplying the ratio of formula 6 by $1/s$, yielding

$$\mu = \mu_m 1/(1 + K_s s^{-1}) \qquad 7.$$

On the grounds that the exponent of s is not necessarily unity as it is in Monod's equation, he substitutes the symbol χ and demonstrates a means of evaluating it. This modification seems justified as an aid in curve fitting even though one does not gain an intuitive feeling for its meaning. Contois (17) also questions the validity of the equation and offers an alternate expression. He proposes that "cell space" may be a factor in controlling growth rate. Moser's analysis provides a tool for quantitative comparison of heritable differences between strains of the same species of cells. The use of this procedure is illustrated in his monograph.

In the externally controlled, continuous culture the growth rate is concentration-dependent. As Novick & Szilard (4) point out, it is not dependent on the concentration of the incoming medium but on the concentration in the culture tube. The density of cells is dependent, however, on the nutrilite concentration of the input medium. This relationship can be expressed by the equation

$$N = (a - c)/Q \qquad 8.$$

where N is the number of cells, a the concentration of nutrilite in the incoming medium, c its concentration in the growth tube, and Q the amount of the factor required to produce a single organism. In most cases, c is very small compared to a. The order of magnitude of c can be appreciated by referring to the half maximum growth factor concentration, K_s, for specific limiting growth factors found in the studies mentioned. *M. tuberculosis*, *E. coli* *Bacillus subtilis*, and *Salmonella typhimurium* on glucose show a K_s from

about 20 mg. to 2 mg. per liter (18); while *E. coli* strains with limiting tryptophan show values in the range of 10^{-3} μg./ml. (4). Parenthetically, the value of this constant would be expected to be different from species to species or even between strains, and it is not surprising to find a large quantitative difference depending on whether the substrate is the sole energy source or is merely a supplement. The concentration of the nutrilite in the incoming medium can be used to estimate Q if the value of $(a-c)$ is well approximated by a alone (5). The value of Q is relatively constant at different growth rates (4, 19); although it appears to be changed at low growth rates.

Since Q is the amount of a factor required to produce a single cell, it can be of considerable importance in determining the effectiveness of substrates. For this purpose one could use Monod's function (18), the yield constant which we will designate as Y, to express a reciprocal relationship although it is stated on a weight rather than a per cell basis. Y is the weight of bacteria produced per weight of substrate utilized. Moser (9) offers a good basis for comparison by expressing the two functions as differentials:

$$dc/dt = -QdN/dt \quad \text{or} \quad Q = -dc/dN \qquad 9.$$

where N is cell number, c is nutrilite concentration, and t is time. Monod's yield constant Y is expressed as

$$dx/dt = -Yds/dt \quad \text{or} \quad dx/ds = -Y \qquad 10.$$

with x being the weight of bacteria and s the weight of substrate utilized.

One of the most informative reports on the theory and operation of continuous cultures is a paper by Herbert, Elsworth & Telling (20). They have developed the theory in such a lucid and concise fashion that it provides an excellent introduction to this field. In it are set forth the equations originally derived by Monod (16) with a minimum number of inductive leaps in the development. For example, from the equations for the change in cell concentration with time and the substrate concentration with time, the steady-state values are derived. The solution to these fundamental equations are then presented in a graph showing the theoretical relationships between dilution rate (washout rate) and each of the steady-state values of substrate, bacterial concentration, doubling time, and output of bacteria. Furthermore, the relationship between the output of bacteria per unit time (the output rate) and the quantity of bacteria produced per unit weight of substrate (the effective yield) is derived. Using these parameters they draw a comparison between continuous and batch cultures in terms of output. The continuous culture was shown to have an output advantage of from five to ten times that obtainable through the operation of batch cultures. Their experimental work with *Aerobacter cloacae* shows good agreement with theory. The device used in these experiments was relatively large and in it they observed several anomolous "apparatus effects." Generation times shorter than the minimum found in batch cultures were observed and were attributed to lack

of homogeneity in the culture, probably resulting from wall growth. There was a lack of mutation to short generation time organisms such as observed by Novick & Szilard (4).

Early Work on Continuous Cultures

Up to this point we have addressed ourselves to the major advancements in theory and operation of continuous cultures that have occurred in the last decade and a half. In this sense we have neglected much of the groundwork that has been important in showing the need for these techniques. If one looks at the previous literature, there are a host of investigators who recognized the value of such systems. The subject of one of the original papers in the field was on the virulence of an organism as a function of growth conditions (21). The continuous culture method provided a partial answer to this question.

The motivation for the construction of continuous culture devices appears to have changed with the development of microbiology. The first instruments were designed primarily for the production of organisms to avoid the bother of constant subculturing (22) and gave little attention to any type of formulation of the rates of growth or flow. With the gradual realization that the phase of a batch culture plays a considerable role in determining the characteristics of a given organism in terms of its capacity for lactic acid formation or glucose utilization, the necessity for continuous systems became evident (23, 24). It was the emphasis on the limiting conditions in the stationary phase of batch cultures that led to the studies by Clifton *et al.* (25, 26) on growth requirements as they affect population size, oxidation-reduction potentials of cultures, and the production of growth inhibiting factors. Their operation of a continuous culture permitted them to conclude that the main reason for the cessation of growth was the depletion of nutrilites. The stationary "resting cell" was thus differentiated from cells in logarithmic growth. Most of these systens were operated at flow rates that would now classify them as externally controlled devices.

Logarithmic growth was gradually adopted as a standard condition for sampling cultures. In it cells are presumed to be in a constant state. An added refinement for insuring this constant state is to specify the population density of cells at the time of sampling. Therefore, it is not surprising that attempts would be made to achieve this condition by utilizing continuous culture techniques. Perhaps the first instrument designed to grow cells at their maximum rate was that devised by Myers & Clark (2). They developed a system using a photocell to regulate flow rate. The steady-state obtained by this arrangement was good to within 1 per cent. Myers' work on green algae such as *Chlorella*, *Euglena*, and several species of blue-green algae, together with that of his co-workers, has set a pattern which cell biologists would do well to follow (27 to 32). His experiments with algae have controls and determinations that are often neglected by other workers.

Chemically defined medium was used and cell growth was always evaluated by measuring both the changes in cell number and the dry weight change per unit culture volume. This permitted him to make statements about differences between the "average cells" from one experiment to another. Consequently, his experiments take on meaning over and above work which refers to changes of weight per unit volume of culture or dry weight of the cell sample. These studies of photosynthetic organisms have netted results on oxidative assimilation in relation to photosynthesis, gas exchange, $CO_2:O_2$ ratios, nitrogen metabolism, the effects of temperature and light intensity on photopigments, i.e., chlorophyll and phycocyanin per "average cell," and a host of other properties which are regulated by the cell in response to environmental conditions. Aside from the direct meaning this work has in plant physiology and photosynthesis, one general principle that can be derived is that average cellular properties can be varied by controlled environments.

STEADY-STATES

Although it is perhaps impossible to find a statement in the microbiological literature which specifically maintains that cellular properties are invariant, much of the literature gives one the impression that bacteria and other cells are considered to be rather fixed entities that can be dealt with much as a physicist deals with an atom or a molecule. The reviewer does not presume to maintain that anyone believes this in the real sense but rather that it is a model built through neglect to attack the total problem. It undoubtedly arises from the fact that in dealing with microorganisms one must deal with a population. The constancy of its growth rate in the logarithmic phase is misleading and suggests uniformity of the subunits. This is further accentuated by our recognition of the constant genetic properties of a cell. The tendency has been to regard the cell as a model unit whose functions are invariant in time. To some degree, the use of continuous culture techniques tends to perpetuate this simplification by virtue of the necessity of reducing such studies to the kinetics of numbers and populations. One must be cautious in the interpretation of the steady-state that is displayed by a continuous culture. An accepted definition of a steady-state is that it is a condition of a system such that its composition remains invariant with respect to time in spite of a flux of energy or materials through it. A continuous culture fits this definition but the units of the population, namely the cells or microorganisms, do not, since the growth of each unit in time does not fit the invariance criterion. The growth of a cell between divisions might more appropriately be defined as a transient, albeit one with a relatively long duration.

The idea that a cell can attain an ideal physiological state and the belief that it reaches expression in logarithmic growth has been an argument used in favor of continuous culture apparatuses that function at maximum growth rates. In the symposium edited by the Czeck academician Ivan

Malek (6), this idea is expressed by several of the contributors. Malek's (34) use of the term "physiological state" implies a significance that is not subject to rigorous definition but represents some illusive ideal condition. It is difficult to understand some of the distinctions he makes although it must be said that his paper is a thoughtful consideration of many of the problems and assumptions inherent in this subject. Logarithmically growing cells, because they are saturated with all their requirements, are operating under a condition where none of their exogenous requirements are rate limiting. We must, however, appreciate that the cell properties may still be a function of the environment in which they are maintained. One is not justified in assuming that two cultures both grown at a maximum growth rate but at different levels of nutrilites above saturation will contain comparable cells. To do so implies that generation time or growth rate is the only criterion for the comparison of cells. The question is, whether or not the cell regulates against an overabundance of a given requirement and by so doing is altered. But, to answer questions of this kind which often have their foundation in changes in the population structure or changes per cell, one must be aware of the source of the changes that have occurred.

Age and Generation Time Distributions

The work of Powell (8) on the statistical composition of bacterial populations is pertinent to this problem. Age and generation time distributions in a variety of bacterial species as it applies to batch cultures and continuous cultures, have been one of his major concerns. The variability within bacterial populations that he has analyzed by the techniques of mathematical statistics is an important prerequisite to defining both the population properties and the properties of the individual cells. Even the age distribution of an "ideal" population, that is, one in which all the cells have a fixed generation time, is often neglected in considerations of population kinetics. The number of cells at each age from zero to one generation time decreases with age of the cells, and one finds that during logarithmic growth the distribution will contain twice as many new young cells as there are terminal old cells. The change in number in each age class with age is a decreasing logarithmic function. This arises from the fact that a young class as it ages remains constant in number for one generation time while at the same time there will be a logarithmic increase in the total population. Thus, the proportion of cells in any age class declines with age. Powell shows that this ideal distribution is further complicated by the fact that the generation times are not the same for all cells in the population, but show a definite dispersion. Consequently, the real age distribution is complicated by this spread.

In terms of population structure, the difference between batch and continuous cultures arises from the tendency of a continuous culture to select against cells with long generation times. This is intuitively apparent when it is remembered that the growth rate and the washout rate are interdependent variables. The washout rate determines or is determined by

the growth constant as it in turn is related to the mean generation time. Those cells with generation times longer than the mean of the population would be expected to be washed out if there were a strong mother-daughter correlation with respect to generation time. Over and above this relationship between generation time and washout rate, the time that a cell spends in the growth tube relative to its probability of contributing to the increase in cell number by division during its residence, must be taken into account. Powell concludes that mother-daughter correlations in a continuous culture can be neglected at our present level of knowledge. He also states that under limiting nutrilites the frequency function of the generation time must alter in scale if not in shape. He is not of the opinion that under limiting conditions viability will remain near 100 per cent. In another paper (35), he considers contaminants and mutants in continuous cultures and gives the conditions which will lead to their removal from or domination of the culture. These findings force us to qualify our classical determinate model of the cell by including in it some measure of variabilty. For the sake of realism, we must recognize that the range of a cellular property, although governed by genetic limits, is considerable even in a well-controlled environment. Variability of itself is a property which may endow an important selective advantage on any species of cells (36). It is only by the use of continuous culture procedures that one can properly separate the intrinsic variability of a cell from that superimposed upon it by the fluctuating environment inherent in batch culture techniques. Even the intrinsic variability of a cell may have several sources. Population properties averaged over a population of cells cannot be regarded as arising solely from a quantitative change per cell since the time of appearance or synthesis of a constituent in the cell cycle could result in different population averages in light of the age distribution. This could occur even though cells from two populatons had the same amount of a given constituent at the time of their division. In fact, the average of any constituent which is synthesized linearly over the cell cycle does not represent a cell of mean age but a cell which has completed 41.5 per cent of its generation time (37) when an ideal age distribution is present. If values of the cellular content of a given constituent can be estimated at any stage of its cycle, the above relationship can be used in conjunction with it to guess at its course of synthesis over the cycle, provided the dispersion of the generation time distribution is not so great as to mask the age distribution relationship.

Adaptation of Cellular Properties in a Continuous Culture

In this context, workers have long been aware of several "average" gross cellular properties that are a function of the cell's environment. "Mean cell volume" is one such property which may be a function of growth rate and in turn related to the nutrients, pH, incubation temperature, etc. The mean cell volume is a weighted mean inasmuch as each cell in the population changes its volume over the cell cycle. In this respect, the distribution of

cellular volumes will show some relationship to the age distribution although this may be obscured by several interactions. One will be the dispersion of a volume distribution at any single cell age and the other will be the variability of the cell volume as it is related to the dispersion of the generation time distribution (8). In batch cultures some of these effects are demonstrated by protozoan cells whose mean cell volumes and range of volumes are a function of temperature (38, 39, 40) and of growth phase (41). Yeasts appear to show volume changes as a function of growth rate in continuous cultures (42, 43), while *Aerobacter aerogenes*, *Bacillus megaterium*, and *Staphylococcus aureus* show variation in mean cell mass as a function of growth rate (44). The dimensions of *E. coli* have also been studied (45) as a function of growth rate and there appears to be an independence of the division of chromatin and the doubling of the cell's dimensions. Nuclei per cell and cell mass per nucleus in *Salmonella typhimurium* have been examined by Schaechter *et al.* (46), and they have found changes in size and composition of cells with the growth rate and medium but not with incubation temperature. A lack of synchrony between growth and chromatin division may have an interesting implication when filtration of a population of cells of this type is used to synchronize their division (47). Is there the possibility that chromatin production is not linked to cell division (48)? If so, one must recognize that an apparent linear synthesis of constituents over a synchronized cell cycle (49) could also arise from short synthetic periods being distributed randomly over the age of the cell. The dispersion of cell ages in a population would also tend to randomize the synthesis pattern. Any change in the environment can have a synchronizing effect on a population. Differential rates of synthesis of several constituents with acute medium changes have been demonstrated by Kjeldgaard *et al.* (50).

As yet no one has devised a rule by which to predict changes that occur in the average cellular dimensions with changing growth rate. It is likely that mean cell mass and volume will have a conjugate relationship to each other in any such scheme. Electronic counters and particle-sizing equipment will aid greatly in the accumulation of such information (51) since microscopic data on small cells are difficult to obtain because of the limited resolving power of the light microscope. The yield constant is also a function of these gross cellular dimensions and the similarity or dissimilarity between cells under different conditions can be evaluated by such determinations. Substrate seems to have little effect on the yield constants of yeast and *S. faecalis* (52) grown under anaerobic conditions and at the same growth rate.

The dependence of cellular respiration on cellular dimensions is also an area open to question since few data are presented where respiration rates are expressed per cell. Oxygen requirements have been examined in continuous cultures of *A. cloacae* by Pirt (53) who found that output of various metabolites is a function of the oxygen available. The O_2 consumption is a linear function of the growth rate of *A. aerogenes* in a continuous culture when expressed per milligram dry weight per hour. Carbon dioxide liberation

has also been examined (44). Inasmuch as the mean cell mass triples at the high growth rates, one must conclude that at high growth rates respiration rates per cell are much higher.

The respiratory machinery of cells may also be affected by the growth rate. Chaix & Petit (54) have measured the cytochromes at different growth rates in *B. subtilis* and have found differences. Rosenberger & Kogut have carried out similar studies in a fluorescent pseudomonad (55). When oxygen or succinate is made limiting, growth rate decreases give rise to an increase in cytochrome. Although both limiting factors produce an increase in these compounds, oxygen limitation doubles the cytochrome level relative to succinate limitation. Both determinations have been carried out at the same growth rate.

Oxygen tension is very critical in the continuous culturing of tissue cells. Cooper *et al.* have shown that excess O_2 tension as well as lowered levels will limit the growth of embryo rabbit kidney cells (56). These authors report the development of a turbio-chemostat for carrying on continuous cultures of tissue cells.

Hemolytic streptococci have been grown under pH-limiting conditions by Karush *et al.* (57). They propose that to obtain high yields of a specific end product, attention must be paid to the type of nutrilite that is made limiting, since maximum cell yield does not of necessity provide maximum end product yield. These are important factors to consider not only in regard to theory but also with respect to industrial applications.

Metabolic studies using continuous culture techniques are often hard to interpret when defined media are not used. However, such studies are of great value in commercial microbiology (42, 58 to 62). The output of specific products should be examined under defined conditions such as used by Rosenberger & Elsden on *S. faecalis* (63) or by Neronova & Ierusalimskii (64). Basic work of this kind can be expected to provide rules for the operation of industrial systems. The introduction of series cultivators is a promising innovation in this area, and Maxon's theory on the operation of these fermentors lays a foundation for their use (65). The desired end can sometimes be best accomplished by attention to either cell content (66) or to metabolic by-products (57). Immunogenicity and virulence is another area of importance (67), and attempts have been made to induce bacterial antagonism between *Proteus vulgaris* and *E. coli* but without success (68). The ecological interaction of two species of bacteria has been studied earlier (69).

As Monod (3) predicted in his original paper on continuous cultures, they are ideal systems for the investigation of the kinetics of formation of cellular constituents. Ribonucleic acid, DNA, total proteins, and enzymes all fall into this category. Ribonucleic acid synthesis as a function of growth rate has been a subject of some concern and was first examined in continuous cultures by Jeener (70). He grew the protozoan *Polytomella caeca* and showed that the RNA:protein ratio increased as growth rate increased when either phosphate or acetate were made limiting. In batch cultures of *Chilomonas*

paramecium where growth rate is controlled by incubation temperature the same relationship is found (40). The question of how RNA content per cell changes at different growth phases of a batch culture is not easily answered since the time to reach a constant amount per average cell is an adaptation which may not appear with acute changes in the growth rate. Continuous cultures are a means of removing this doubt. Differences do appear between lag phase and log phase cells (49), but in *E. coli*, Hedén *et al.* (71) found no change in adsorption at 260 mμ with growth rate. Herbert's data on *A. aerogenes* (44) show that the percentage of RNA does increase with increasing growth rate. In the same experiment, the percentage of DNA and protein decline slightly with increasing growth rate. If expressed per cell, there appears to be about seven times more RNA in a fast growing cell than in a slow growing cell. The ratio of RNA to DNA or protein shows a definite increase with increasing growth rate. The slowest growing cells correspond closely to resting cells.

The study of enzyme formation under specific growth conditions is intimately linked to induced enzyme formation. This subject is far too extensive to review in this paper, but a few applications of continuous culture methods can be mentioned. The variation in enzyme activity as a function of the growth conditions under which a microorganism is maintained may not fall into this category although in some instances the distinction may be one of degree rather than of kind.

Davies has used continuous cultures of *Saccharomyces fragilis* to examine the conditions under which invertase (72) and lactase (73) activity appear. The formation of invertase is markedly inhibited by glucose in the culture medium. It was shown that its appearance was not related to the mean generation time of the organism. This was done by the use of well-designed controls in which various limiting growth factors were employed. A similar pattern is seen for the formation of the enzyme lactase. Continuous cultures of *beta*-galactosidase-induced *E. coli* have been used by Cohn & Torriani (74) to study the antigenic properties of proteins in induced and uninduced cells. These were compared for cross reactions and an uninduced protein was found to be comparable in antigenic properties to the enzyme protein. Many of the studies on this system have been performed without the aid of such devices and one cannot argue that steady-state cultures are always necessary to such studies. However, investigations by Novick & Weiner (75) in which the Chemostat was employed, have led to results that might not be easily obtained by other methods. In their work they were able to follow the kinetics at various levels of "gratuitous induction" and show that the induced cells have a reduced growth rate which accounted for the loss of activity by the partially induced systems. This, plus other data in their investigation, led to their proposal that induction at the level of the cell was an "all or none" phenomenon. Using similar culture techniques, Gorini & Maas (76) have provided an excellent example in the ornithine to citrulline system of *E. coli* of how the end product, arginine, of a biosynthetic sequence

inhibits enzyme synthesis involved in the formation of the product (77). Novick's investigation of the effect of exogenous arginine on the regulation of arginine synthesis in *E. coli* is another case of the control of enzymatic systems (78). He showed that the cell's synthetic production of arginine was depressed by exogenous sources of this amino acid. The formation of an ultraviolet-absorbing compound which is released in to the medium by an *E. coli* mutant, B/1 t, at low concentrations of tryptophan and long generation times have been reported by Novick & Szilard (7). The compound is thought to be indole-3-glycerol phosphate which is an intermediate in a biosynthetic pathway to tryptophan (79). Its accumulation at growth-limiting tryptophan concentrations in a tryptophan-requiring mutant suggests a situation comparable to end product control over biosynthetic pathways since it is synthesized most rapidly at low tryptophan concentrations.

Genetics and Evolution in a Continuous Culture

Novick & Szilard's introduction of the Chemostat (4) was accompanied by several interesting discoveries that were concerned with the genetics and evolution of microorganisms. They used the B strain of *E. coli* and tested for resistance to the T5 phage as a means of identifying mutants. Early in their work they found that a changeover from strain B/1 to a faster growing strain occurred under the conditions of limiting tryptophan. They designated it B/1 tf and showed that it grew about five times faster than strain B/1. This experiment is central to the study of bacterial evolution as it illustrates how short generation times are selected for by a continuous culture. Furthermore, they developed equations covering the changes that occur when spontaneous mutations are generated in a growing culture with growth rates the same as the parent strain so that there will be no selection for or against it by the washout rate. Northrop & Kunitz (80) have developed equations of a similar kind, and Northrop (81) has applied them to studies of terramycin-resistant mutants of *B. megaterium*. In the case where growth rates are the same for the mutant and the wild-type, the relative abundance of the mutant will increase linearly with time. In the case where the mutant strain has a growth rate that is less than the parent strain, after the initial rise, the number will remain constant. Moser (9), in his monograph, covers these situations in mathematical detail. He is concerned with precise definitions of specific and non-specific selection as they apply to the populations in a Chemostat as well as mathematical definitions of other parameters of selection. This monograph covers a variety of situations that are pertinent to population structure and change-over, and it does so with such perspicacity that it is not possible to do more than mention it as a significant contribution to this area of study.

Novick & Szilard (82) further extended their work to the measurement of rates of mutation to T5 phage resistance in a Chemostat as a function of growth-limiting tryptophan concentrations, as well as several other growth

factors. It was found that mutation rates expressed per hour per cell were constant over a fairly wide range of growth rates. This finding was curious in the light of the fact that spontaneous mutations appear to be absent in resting cells (83). If spontaneous mutations are dependent on growth they should vary wlth the growth rate. Lee (84) showed that at very low growth rates with tryptophan-limiting, mutation rates to T6 resistance were not constant, but in the same range of growth rates reported by Novick & Szilard it was constant. Demerec *et al.* (19) showed that under nitrogen-limiting conditions, mutation rates were proportional to growth rates in the region of rapid growth but constant at lower growth rates. Mutation rates at maximum growth rates have been studied by the use of an internally controlled system (85, 86), and these studies also support these findings. There is no conflict in these findings since the differences occur in different ranges of growth rate. One hypothesis which might explain these results was offered in the paper by Novick & Szilard (82) in which the "error" hypothesis of mutations was proposed and tested by use of limiting phosphate and nitrogen on the assumption that the rate of gene synthesis was the controlling factor in mutation rates and could be varied by limiting the essentials to its synthesis. The results of such tests showed little or no effect on the rate of mutation. The idea that it might hinge on some cellular rate other than growth rate, for example, the rate of DNA synthesis, is still intriguing since the rate of DNA synthesis may not be linear with cell age in this region of growth rates. If its synthesis occupies only a brief portion of the generation time but its rate of synthesis was constant with a changing generation time, these conditions would occur since limitation of nitrogen and phosphate may not exclusively affect DNA synthesis. In other regions, i.e., high and very low growth rates, the rate of DNA synthesis would have to be proportional to the generation time to give the effects observed by Lee and Demerec *et al.*

Research on the effect of mutagenic agents and growth factor limitations on mutation rate in the B strain of *E. coli* proved that the choice of limiting factors and mutagenic agents could have a profound effect on the mutation rate. In fact, antimutagenic substances were also found (87). Guanosine and other nucleosides were effective in reducing mutation rates to levels less than those found occurring at spontaneous rates although a phenotypic lag of about 12 hr. was shown to exist. Thus, we see that the combined use of mutants and the Chemostat has been very successful when applied to the physiology and biochemistry of microorganisms.

Steady-State Populations Versus Synchronized Populations

We have attempted to emphasize the importance of examining the cell as an integrated entity rather than the culture. We feel that the reduction of data so that it is expressed per average cell yields information that can be more meaningfully interpreted in terms of cell function than data that are expressed as percentages of dry weights or unit culture volume. The continuous culture technique is undoubtedly the most effective means of attack-

ing a variety of cell problems since it frees the system of the variability that accompanies changes in growth rate and nonsteady-state environments. It is at the same time a means of insuring a stable statistical sample of cells which, barring mutations, can serve as a baseline for comparison with cells under other controlled culture conditions. At first glance, it might be assumed that it can provide little information about the operation of the cell cycle but this need not be true since careful attention to the population structure, i.e., age structure and generation time distribution, may yield presumptive information on the course of events in the life cycle of a micro-organism. In this sense we should be grateful for the non-random structure of the age distribution since it may be the chink in the statistics of cell populations that will lead to knowledge about the cell cycle from continuous culture populations.

To those of us who are concerned with the cell cycle and the physiological and biochemical processes that lead to cell division (88, 89), the fact that all cells in a population, each of a different age, can each behave uniformly in the synthesis of certain induced enzymes is not a startling fact. These can be relegated to the realm of systems that are not of first importance to the division process. At the same time, it is recognized that all subcellular systems cannot behave in this manner since some heterogeneity in time must appear in the growth and division cycle.

The use of various "abnormal" means of trying to achieve a population in which the age of the cells is not random but confined to a narrow class, does not exclude the use of cells from a normal random continuous culture as the best baseline. Even if one grants that certain synchronization methods distort the normal cell cycle, they at least yield a population in which the cell divisions are confined to a narrow region in time. The synchronization of cells in a culture can be used in conjuction with other treatments in which dilution at some point in the generation time is programmed to the cell cycle (90, 91). In such systems, the synchronizing treatment as well as the dilution must be in phase with the cell cycle. Controlled but cycled environmental changes may be the key to obtaining cells that will yield information on the transient cellular processes which culminate in cell division.

New Frontiers

Let us hope one of the directions that modern cell biology will take is to set down the relationship between cellular or microbial evolution and thermodynamics. By this, I mean the thermodynamics of the cell unit as an open system, not merely the thermodynamics of its subsystems. To do this will require the introduction of new concepts and attention to the ways and means by which biological concepts such as selection, adaptation, and competition can be framed in a rigorous non-teleological language. Continuous culture experiments have illustrated microbial evolution in the most direct sense. The thermodynamic model of evolution of Prigogine & Wiame (92) may be a first step in this direction. Their ideas may be paraphrased as

follows: they contend, first, that selection in the evolutionary sense operates in favor of systems showing minimum entropy production; second, an open system like a living cell, provided it is in the steady-state, shows minimum entropy production; and, third, steady-states are best achieved by systems with internal controls and therefore by systems which are relatively complex.

Thus, there is a tendency toward increasing complexity in the course of biological evolution. It is not our purpose to argue the pros and cons of this statement or to defend their hypothesis but it is important to ask if a logical trace can be drawn between thermodynamic restraints and evolutionary restraints. The difficulty of doing this is apparent to anyone who has attempted to define the efficiency of a cell as reflected by its capacity to channel metabolic energy into various activities. Invariably, a human value judgment is made in which some cellular activity is considered to be of greater evolutionary value than some other. The proof that minimum entropy production is achieved in the steady-state is a first step (93). But, here again, definitions are a source of discomfort for, as previously stated, a continuous culture obviously achieves a steady-state since its composition is invariant with respect to time, but the growth and division of an individual cell cannot be considered a steady-state since the units are not invariant in their composition. Nevertheless, comparison of cells with regard to their properties, should be carried out under the rigorous condition of steady-state cultures such that there is no net change in the range their constituents or activities take so with respect to time. To study entropy production would, of course, require measurements of the rates of input and output of gases, nutrilites, by-products, and heat production, as well as calorimetric evaluations of cellular constituents with appropriate references to the standard states of each. To date, I have not found any attempts to measure heat production in a continuous culture although heat production has been measured in both concentrated (94) and logarithmically growing cultures (95). In the latter cases, these various parameters were taken into account although the purpose of the measurements was not directed strictly to the evaluation of entropy production. It would be hoped that this problem will be attacked in the future, particularly with respect to the differences in entropy production between closely related genetic strains grown under well-controlled conditions of continuous culture techniques.

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FINE STRUCTURE OF PROTOZOA[1,2]

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Perhaps no living system is better adapted than certain protozoa for the study of ultramicroscopic structure. It is not surprising, therefore, that the advent of the electron microscope, together with the development of suitable methods for its use in biological research, led to an intense interest in the study of protozoan fine structure. Investigations in this field have been rapid and informative because of the extensive knowledge of protozoan structure long ago made known by classical researches based on methods of study with the light microscope, and it is interesting to note that, notwithstanding the numerous papers currently appearing which deal with protozoan ultrastructure, few new structures have been revealed. The bulk of the observations so far has been largely confined to a more detailed analysis of well-known differentiated surface and cytoplasmic components. Knowledge of protoplasmic structure per se has been surprisingly little enhanced by electron microscope observations alone. It is only when such data are correlated with those from other sources, such as polarization microscopy, x-ray diffraction, radioautography, cytophysiology, and especially cell fractionation studies, that important advances can be noted in such a complex system. In fact, it has been suggested of late that " . . . it is now widely expected that the simplest form of life will be understood in molecular terms within the next twenty years" (133).

Thus far, research on the ultramicroscopic structure of protozoa has demonstrated that their basic structure is essentially the same, but often more pronounced than that found in certain cells of other organisms; that is, the organization and fine structure of the organelles, such as cilia, flagella, mitochondria, Golgi material, plastids, endoplasmic reticulum, fibrous, and lamellar systems parallel closely those in certain cells of higher forms. Hence, the hypothesis that the protozoa should be considered non-cellular organisms finds little support from a study of their submicroscopic structure. However, there can be no doubt that some protozoa are among the most highly differentiated and specialized cells of all, and the basic molecular structure and organization of their myonemes, cilia, and flagella doubtlessly parallel rather closely those found in muscle of higher animals. But, unlike the condition in muscle, the organelles of protozoa are readily reversible, especially upon division and encystment of the organism. Such conditions afford an excellent opportunity for the study of differentiation, replication, modula-

[1] The survey in the literature pertaining to this review was concluded in December, 1960.

[2] The following abbreviations will be used: DNA (deoxyribonucleic acid); PAS (periodic acid-Schiff); RNA (ribonucleic acid).

tion, and dedifferentiation of the molecular architecture and organization making up such highly specialized systems.

In this discussion an effort will be made whenever possible to correlate the submicroscopic structure with its possible function. However, not until further research is accomplished in this relatively new field can a useful critical evaluation of current results be rightfully made. Thus, this paper, of necessity, must deal more with a general review, rather than a critical evaluation of the literature. Comparisons will also be made between the submicroscopic structure of protozoans and that of other cells when possible and when conducive to a clarification of discussion. For convenience of presentation, it seems desirable to arrange the discussion under headings of the major groups of protozoa.

Sarcodina

Several excellent reviews of the extensive literature dealing with the structure of this group of organisms are available, especially of the *Amoeba*, at the light microscope level of observation (10, 89, 108). Knowledge of this literature is highly desirable as an aid in better understanding and interpreting of the structure of these organisms at much higher magnification.

The nucleus.—The electron microscope observations of Bairati & Lehmann (8) on fragmented portions of the nuclear membrane of *Amoeba proteus* reveal it to be composed of an inner relatively thick, porous, honeycomb-like layer and an outer thin continuous layer, a finding which was soon confirmed by Harris & James (88). More recently, the outer layer has been found by Greider *et al.* (80) and by Pappas (128) to be composed of a double porous membrane much like that limiting the nucleus in cells of higher animals (11, 186). The pores are formed at intervals by continuity between the inner and outer membranes. Pappas (128) describes in some preparations a diaphragm-like structure across the opening of the pore; this has not been mentioned by others studying the fine structure of this membrane (109, 158).

Intimately associated with the outer nuclear envelope but capable of being separated from it by bleb formation, is an inner layer which is relatively thick (280 mμ) and made up of closely packed hexagonal prisms, each of which terminates at the nuclear membrane in a precisely centered pore (79, 109, 128, 131), so that a continuous channel exists from the inside to the outside of the nucleus except for the possible presence of a diaphragm across the pore in the outer envelope. In sections normal to the surface, the inner layer appears to be composed of canals or tubes and, as mentioned above, are arranged in a honeycomb-like manner perpendicular to the outer envelope. Upon division of the nucleus, this thick inner layer disappears. Cohen (36, 37) and Roth (158) suggest that the nucleolar material may be implicated in its renewed formation at the end of the division cycle. This type of nuclear envelope was thought to be unique for *Amoeba proteus* until a similar structure was found in the nuclear envelope of a gregarine [(13); also see (75)].

The nucleus of *Endamoeba blatta* also possesses a two-layered nuclear envelope but, unlike that of *A. proteus* and *Gregarina rigida*, it is the inner

instead of the outer layer which is comparable in structure to the nuclear envelope of other types of cells (16). The inner porous layer is approximately 40 mμ thick and is composed of two membranes. Adjacent to this layer is the outer, relatively thick porous one. Each pore or microtubule in this layer is approximately 450 mμ long and 50 mμ wide; they extend perpendicularly from the surface of the inner layer, thus producing a multitude of passageways within the envelope. It is not known what function or functions the outer membrane serves. In any case, it is evident that an envelope of this nature would not be conducive to an active process of diffusion were it not for its porous nature. Since the compound type of nuclear envelope is not present in certain small *Amoeba*, Mercer (109) thinks its presence may be related to the large size of *Amoeba proteus* and to the non-spherical shape of its nuclei. In *Endamoeba blatta* the outer layer cannot be a product of the nucleolar material as has been suggested for the inner layer of *Amoeba* (158).

The nucleus of the cyst of *E. invadens* is unlike that of the trophozoites; it is surrounded by a zone of dark material showing some evidence of stratification (42, 43).

Immediately beneath the nuclear envelope in *Amoeba proteus* are found a large number of dense bodies which are thought to be analogous to nucleoli (22, 79, 80, 109, 131, 158). Elsewhere within the nucleus Pappas (127, 131) noted Feulgen-positive regions which contain clumps of helical structures. The longest spirals range between 250 and 300 mμ in length, and several helical threads appear to radiate from a common center. The helicoidal structures measure about 25 to 28 mμ in diameter with a distance of 14 mμ between repeating turns. Because of the position of the helicles within the nucleus, Pappas (127) suggests that they may occupy an " . . . intermediate position between the nucleic acid molecules and chromosomes differing from both by an order of magnitude" [also see (109)]. Cohen (36), on the other hand, observed threads of a similar size within the nucleus of *A. proteus* but they were seldom coiled. He suggests that the threads may be nucleolar rather than chromosomal because their total mass greatly exceeds that of the chromosomes seen at the metaphase plate. Roth (158) is uncertain whether or not the helices described by Pappas (131) represents DNA; in any case, like Cohen (36), he thinks they may be of nucleolar origin.

The mitotic spindle forms within the nuclear envelope of *A. proteus* during prophase; at metaphase the thick inner nuclear layer disappears and large gaps or openings appear in the thin outer nuclear envelope, thus allowing a mixing of the nucleoplasm with the cytoplasm (158).

Mitochondria.—The mitochondria which are the most conspicuous components of the cytoplasm are randomly distributed throughout the organism except for the region around the contractile vacuole where they are more concentrated (80, 107, 109, 130). Because of their position and structure, the mitochondria in *A. proteus* are thought to represent the *beta*-granules (80, 108, 109, 130). The internal structure of *A. proteus* mitochondria is similar to that observed in other protozoa (177). They possess an outer continuous membrane and an inner membrane which has fingerlike projections or micro-

villi (cristae mitochondriales) extending irregularly into the mitochondrial stroma (20, 129). In *Peleomyxa carolinensis* the microvilli pattern of the mitochondria is more complex; its form is often zigzaglike with bulbous enlargements at the points of inflection on the waves. They are often unevenly distributed within the mitochondrion, one end containing numerous enfoldings, the other being relatively free; however, the latter often contain masses of fibrillae (129). The significance of this structural pattern within the mitochondrion is not clear. *P. carolinensis* also shows an interesting relationship of the mitochondria to the nucleus; the limiting membranes of the mitochondria and postdivision nuclei are often continuous (23). The significance of this condition is not known but some authors (127, 129) speculate that perhaps the mitochondria renew their RNA supply from the nucleus, or that this condition simply represents a case of the origin of mitochondria from the nucleus. In *Hartmannella rysodes*, mitochondria are often closely associated with lipid droplets (131) as is also the case in different types of vertebrate cells (6, 125).

In the parasitic amoeba, *Endamoeba invadens*, Deutsch & Zaman (42) were unable to demonstrate mitochondria. They suggested that the absence of this organelle system " . . . points to the absence of those enzymes which generate energy for the cell by transfer of electron to oxygen; or the enzyme may be present but dispersed in the cytoplasm."

Golgi complex.—A structure comparable to the Golgi material of higher organisms has been reported for *Amoeba* (*proteus* and *carolinensis*). This consists of groups of tightly flattened vesicles which appear as a series of parallel membranes. Associated with these membranes are vesicles of varying size. Hence, evidence from studies with the electron microscope leaves no doubt that the Golgi material exists in the protozoa (12, 68, 69, 70, 72), but what, if any, relationship this material bears to the so-called vacuome (neutral red-staining bodies) has not been revealed (84, 94). Its function here as in many cells of higher animals is largely unknown.

Endoplasmic reticulum.—The cytoplasm of the larger *Amoeba* (*proteus* and *carolinensis*) is highly aveolar when observed in the electron microscope. Most of the elongated and saclike vesicles are smooth surfaced (80, 131). The small vesicles surrounding the contractile vacuole may be derived by a kind of pinching off process from the larger saclike ones. In *H. rysodes* a "typical" endoplasmic reticulum exists, i.e., a system of flattened vesicles with ribosomes present on the outer surface of certain of the membranes (131).

Contractile vacuole.—The contractile vacuole is limited by a membrane which is said to be comparable in structure to that of the cell membrane, except for the fringe material; this, in turn, is surrounded by a densely packed layer of small vesicles (102, 109, 129). Centrifugal to the vesicular layer is a well-developed layer of mitochondria (109, 129). Pappas & Brandt (129) noted that certain of the small vesicles appeared to empty their contents into the contractile vacuole, whereupon their membranes became incorporated within its walls. As previously mentioned, the vesicles may be derived from the smooth type endoplasmic reticulum by pinocytosis. In fact, Pappas &

Brandt (129) have postulated a mechanism for the segregation of water from the cytoplasm and its accumulation within the contractile vacuole by assuming the small vesicles to be the loci of "solute-solvent segregation."

Bairati & Lehmann (8) observed a layer of fibrillar material surrounding the isolated contractile vacuoles of *A. proteus;* a somewhat similar structure has been described by Greider *et al.* (80) for the same species of organism, and it has been suggested that such a zone of fibrous material may be important in the contraction of the vacuole. In current literature, however, the presence of such a special contractile layer around the contractile vacuole is questioned (109), and further investigation is needed to resolve this question.

Food vacuole.—The food vacuole of *A. proteus* is enclosed in a well-defined membrane like that of the plasmalemma from which it is derived. It often shows tongue-shaped diverticuli which have been suggested as a method of increasing vacuolar surface area, a condition thought to be conducive to a more rapid secretion of enzymes (109). Adjacent to the outer surface of newly formed vacuoles is a layer of highly dense granules. A budding off of small vesicles by pinocytosis from the older food vacuoles has been suggested as a method whereby the soluble products of digestion are removed from the food vacuole to the cytoplasm (109, 156).

Crystals, alpha-*particles, and unidentified structures.*—The crystals of *Amoeba* cannot be observed by the electron microscope; only the clear outline (negative image) of their form is evident (131). This is apparently attributable to the fact that they cannot be fixed and embedded by methods suitable for electron microscope study. *Alpha*-particles also have not been observed in electron micrographs by Greider *et al.* (80) or by Pappas (131); however, Cohen (37) identifies them as small dense bodies located within small vacuoles. The well-known "chromatoid bodies" of *E. invadens* consist of particles about 20 mμ in diameter arranged in crystalline pattern [(9, 42, 43); also see (124)]. Like many other bodies, the significance of these is unknown. However, they resemble in structure certain viral particles or possibly microsomes in a peculiar state of aggregation. From histochemical studies, it was determined that the chromatoid bodies consist mainly of ribonucleic acid and some unspecified proteins (43). The cytoplasm often contains, in addition to the structures described above, many ultramicroscopic vacuoles, lipid bodies, and granules of unknown nature; these may be stratified in the centrifuge [(40); also see (97, 132)].

Plasmalemma.—The plasmalemma of *A. proteus* has been represented as a double membrane with a variable thickness (36, 80, 102, 103, 109, 131). Externally it usually possesses a fringe of fine wavy filaments. *A. carolinensis* also possesses a similar type of structure in its plasmalemma (158), but this is not the case for smaller species such as *Hartmannella rysodes* (131). The fringelike layer disappears upon division of the organism. The structure of the plasmalemma of the larger amoebae suggests that in addition to serving as a semipermeable membrane it functions to shield the organism from mechanical injury, to increase its absorption surface, and to aid in its locomotion. It is known to give a positive periodic acid-Shiff reaction (126).

The pseudopodia of Heliozoa are axiopods consisting of a stiff axial rod covered with a layer of streaming protoplasm. The fine structure of the axiopodia of *Actinophrys sol* has been investigated by Wohlfarth-Bottermann & Krüger (191). They found the axial rod to be composed of longitudinally arranged ultramicroscopic filaments. Similar observations were made by Anderson & Beams (5) on the axiopodia of *Actinosphaeriun nucleofilum*. Here the axiopodia revealed mitochondria with tubelike cristae, tubular endoplasmic reticulum, and vacuoles containing dense particles in the cortical layer. The axial rod is made up, in part, of filaments of varying lengths which are oriented parallel to the long axis of the axiopodia, and appear to end freely in the endoplasm in the region of the nuclei. Polarized light studies also indicate the presence of oriented ultramicroscopic elements in the rods of the axiopodia [(5, 150); also see (99)]. Perhaps because of technical difficulties, organisms of the order Foraminifera have not been investigated with the electron microscope but have been used recently in a beautiful study of protoplasmic streaming by Jahn & Rinaldi (95).

A study of the ultrastructure of *Plasmodium lophurae* has revealed that, in addition to a diffusion of food materials from the host, the intracellular parasite engulfs host cytoplasm by an invagination of its plasma membrane with the subsequent formation of food vacuoles. This process has been termed "phagotrophy" and is probably a common phenomenon among malaria parasites, since it has also been found to occur in *Plasmodium berghei* (171, 173). In the latter organism, the food vacuoles are surrounded by numerous dense pigment (hematin) granules. These bodies which are enclosed in vesicles are thought to be derived by a "pinching off" process from the food vacuoles.

Typical mitochondria were not observed in *Plasmodium berghei* (173); however, bodies composed of concentrically arranged double membranes are present which may represent the mitochondria. The membranes of these bodies are sometimes continuous with both the double membrane of the food vacuole and plasma membrane. Present also are elements of both the rough and smooth type of endoplasmic reticulum.

Malarial oöcytes (*P. cathemerium*) are encased in a relatively thick capsule which becomes thinner and in places completely disappears at the time of release of the sporozoites (47, 48). The capsule is thought to be a product of the host rather than of the parasite and it may be shed into the oöcyst where it is digested and utilized by the parasite. In sporozoites certain unidentified rod-shaped bodies of moderate density are present. Still unsolved is the important question as to how the contents of the oöcyst are transformed into individual sporozoites. Both endoplasmic reticulum and mitochondria are present in oöcysts and sporozoites (47, 48). However, mitochondria are absent in merozoites (113).

Knowledge of the ultrastructure of *Toxoplasma* has been considerably enhanced by means of the electron microscope, especially the nature of its locomotor organelles. At the anterior end of the parasite there is a short truncate hollow cone, designated the "conid" (24, 83). The base of the conid

is in contact with the cytoplasm and the distal end is closely associated with the cell membrane. Extending from the proximal end of the conid toward the blunt end of the cell are 14 to 18 homogenous fibrils ("toxonemes"); these are thought to be unique for *Toxoplasma* (24). The conid probably functions as a mouth structure, or penetration device, or it may be a vestigial cytopharynx. Its movement may be caused, in part, by the contraction of the toxonemes. A fibrillar network located on the surface of the body has been suggested as the locomotor mechanism for the gliding movements of the parasite (24).

Electron microscope studies have revealed that the striations in the cyst wall of *Sarcocystis miescheriana* have a honeycomb structure giving rise to many parallel fingerlike villi (106); these contain fine double filaments. The parasite shows a polar ring with a conoid at the anterior pole where 22 fine fibers arise from the polar ring and extend posteriorly. Three different zones can be distinguished in this organism: an anterior fibrillar one containing "sarconemes," a middle granular one, and a posterior area containing the nucleus. The function of the sarconemes remains unclear; they may be contractile.

Gregarines (15, 101) display numerous ultramicroscopic folds in their surface layer. In *Gregarina rigida* the surface layer is composed of three membranes; the outer and middle cover the surface of the folds and the inner one continues as a smooth layer covering the ectoplasm of the animal (15). The outer folds probably function as a device to increase the absorption surface of the animal and to aid in its locomotion. In the ectoplasm is observed a system of anastomosing fibers, some of which appear to be attached to the surface layer (15). Present also is a netlike system of anastomosing tubules; certain of these appear to connect with the surface of the organism through small pores. Possibly they function in the excretion of mucus. However, there was no evidence of posteriorly directed tubules of the type which might be used for conveying mucus to the surface in a manner suitable for supplying the chief motivating force involved in the gliding movement (15). Ultracentrifuged gregarines have the materials of their endoplasm displaced in the order of their relative densities; this condition does not appear to interfere with their locomotion (15).

The endoplasm of both the protomerite and deutomerite is of a highly vacuolar and granular nature (15, 76). A sharp demarcation in structure exists between the endoplasm and ectoplasm; the latter is relatively free of vacuoles and is granular and contains an ectoplasmic network of fibers and tubules. Present also in the inner portion of the ectoplasm are myonemes. The septum separating the endoplasm of the protomerite and deutomerite is a thick membrane; only in the ectoplasm does the protoplasm of the protomerite and deutomerite appear continuous (15).

Sections through animals in syzygy reveal that the surface folds of the two associated animals are in direct contact. No morphological connections exist between the gregarines in this region and they seem to be held together by a mucoid substance (15).

There is no question that *Gregarina rigida* spends part of its life cycle within rather than between the epithelial cells. Sections through the caeca of grasshoppers (*Melanoplus differentialis*) parasitized with *G. rigida* often show organisms in various stages of growth within the long epithelial cells [Beams & Anderson (19)]. Even in the very early stages, the parasite has a definite surface membrane which isolates it from the surrounding cell cytoplasm. As it develops a portion of the body extends through the microvilli-covered surface of the cell. At this and later stages, the host cell develops an invaginated smooth surface membrane which is now in close association with the surface of the parasite. This membrane relegates the parasite to an extracellular position [Beams & Anderson (19)]. However, during the early growth stages, it is surrounded by the host cell membrane in much the same manner as that which exists between young differentiated mammalian sperm and Sertoli or nurse cells (27).

Mastigophora

Pellicle.—Electron micrographs of *Euglena gracilis* display in detail the nature of the grooves and ridges; the semiridged rings alternate with soft pliable membranes (85, 86, 148, 197). Sometimes (as in *Peranema*, *Euglena*,) the ridges are modified to form a groove in which the trailing flagella lie (86, 155). Whole mounts of osmic tetroxide-fixed and air-dried pellicles of *E. gracilis* and thin sections of *E. viridis* show the longitudinal spirals to possess paired fibrils about 25 to 50 mμ in diameter; these are composed of smaller subfibrils. *Peranema* also shows a system of subpellicular filaments running parallel to the pellicular ridges (155). Subpellicular fibers are also present in *Trypanosoma mega* crithidias (182).

The striated appearance of *Lophomonas striata* has been shown to be caused by the presence of bodies (probably bacteria) attached to its highly folded surface (17). The "bacteria" located in the troughs of the folds convey the false impression under the light microscope of being located intracellularly.

The surface of *Trichonympha* is highly differentiated in the region of the rostrum. Here, it is composed of at least four layers: (*a*) an outer double membrane constituting the cell envelope; (*b*) a layer composed of two parallel sheets of parallel fibers; and (*c*) an inner, less regularly arranged layer of fibers (136).

The ultrastructure of tests such as the vase-shaped lorica of certain flagellates (*Bikosoeca*) reveal an orderly arrangement of transverse lines (149).

Nucleus.—In some flagellates, the nuclear envelope resembles typically that of nuclei from cells of higher organisms (11, 186). It is often composed of a double membrane and in *Tritrichomonas* (2, 4), *Trichonympha* (82, 136), and *Chlamydomonas* (174) the membranes join delimiting pores at regular intervals. Evidence of the presence of a thin membrane (diaphragm) across the pores was noted (174). Little new information has been added to our knowledge of the internal structure of the nucleus from studies with the electron microscope except for certain species of Dinoflagellata (74). Here,

the chromosomes are made up, in part, of bundles of fibers (chromonema) arranged in spiral fashion (major and minor coils). We are indebted to Cleveland (35) and others for important studies with the light microscope on the division mechanism of these organisms.

Surrounding the nucleus of *Lophomonas blattarum* is the so-called parabasal apparatus. Studies on the fine structure of this body reveal it to be unrelated to the parabasal body of other flagellates; instead, it is made up of tubes (perinuclear tubules) which radiate from around the nucleus. Each tubule consists of a distal or body portion which narrows abruptly to a highly convoluted tortous and conducting portion which penetrates the calyx membrane; a calyx portion which is highly twisted and shows some anastomosis of tubules, and a relatively dense, junctional portion which appears to perforate the nuclear envelope (18). Thus, here is seen a nucleus with evaginated tubular-like surface structures extending through the calyx membrane into the extracalyx cytoplasm. In structure, the tubules are not unlike microvilli found on the surface of some types of epithelial cells (6, 14). Since the nucleus is located within the calyx and somewhat isolated from the extracalyx cytoplasm, the modification of its surface may be an adaptation for bringing nuclear surface into more direct contact with extracalyx cytoplasm. The nucleus, calyx, and perinuclear tubules seem interlocked and at times may rotate together within the living organism. The perinuclear tubules do not seem to be directly related to the endoplasmic reticulum, unless perhaps they have been modified here beyond any previous conception of structure for the endoplasmic reticulum. This seems true in spite of the fact that in nuclei of *Chlamydomonas* (174) and higher organisms the endoplasmic reticulum may sometimes be continuous with the outer nuclear envelope.

Stigma and paraflagellar body.—In *Euglena*, the stigma is composed of numerous, dense homogeneous bodies containing carotenoid substance (63, 78, 85, 86, 91, 198, 199). The gross arrangement of the granules is somewhat comparable to the grana in the chloroplast, and Wolken (198) suggests that the "eye spot" granules produce a movement of the organism toward a specific part of the spectrum. The function of the stigma has been compared to photoreceptors of higher organisms. Gibbs (78) finds in *Euglena gracilis* that the stigma is located outside the chloroplast with its granules loosely packed; in this respect, it differs from the diagram of Wolken (198). In *Chlamydomonas*, the stigma is composed of two plates of dense granules which are embedded in the matrix of the chloroplast, usually near the surface membrane (174).

The plano-convex paraflagellar body of *E. gracilis* is located within the limiting membrane of the flagellum at a point just below its bifurcation (78); it is considered to be a photoreceptor (197).

Flagella and basal bodies (kinetosomes).—In *E. gracilis* the flagella arise from two "roots" at the bottom of the ridged reservoir [(60, 78, 85, 86, 197); also see (164)]. Each is surrounded by a membrane and possesses the usual nine peripheral and two central fibrils. This arrangement and number of

fibrils (nine plus two) seem to be of general occurrence within both plants and animals, with the possible exception of bacteria (187). Around and extending from the surface of the flagella are many fine filaments (mastigonemata) which probably provide extra resistance for their movement (25, 26, 134). In trichomonads, the anteriorly directed flagella, together with the undulating membrane, recurrent flagellum, "accessory filament," costa, parabasal filament, parabasal body and axostyle, were thought by light microscopists to arise from an anteriorly placed structure called the blepharoplast. Ludvík (105), studying whole mount preparations of *Trichomonas foetus*, reported the ultrastructure of the blepharoplast as consisting of six granules surrounded by a relatively dense cytoplasm. Electron micrographs of sections through the blepharoplast complex of *Tritrichomonas muris* reveal it to be composed of four kinetosomes, one for each flagellum (4, 7).

In *Lophomonas striata* and *blattarum*, the flagella number over 100 and are arranged as a tuft on the anterior surface of the calyx (16, 18). Each flagellum ends in a relatively long and clearly defined basal body, which is apparently connected to the other basal bodies by two fibrous membranes, one located about 250 mμ below the surface of the calyx and one attached to the proximal ends of the basal bodies. Extending from the latter is a delicate network of fibers; these may serve to help coordinate the movements of the flagella. In *Peranema* the flagella possess, in addition to the usual 11 filaments, the following structures: an intraflagellar rod, and a layer of material on the outside of the membrane. Near the basal region of the flagellum a swelling occurs in the shaft which is possibly correlated with the absence of the two central fibers in this region (155).

Perhaps the most complex flagellar structure of all is found in *Trichonympha* and closely related organisms. Pitelka & Schooley (134) were the first to examine these organisms with the electron microscope, and they observed that flagella arose from the endoplasm deep within the cell, but apart from the centrioles. Each flagellum traverses a thin layer of the endoplasm and a thick, clear ectoplasm, to emerge from the cytoplasm at the base of the deep, longitudinally arranged surface grooves. More recently, Gibbons & Grimstone (77) have studied the ultrastructure of *Trichonympha*, *Pseudotrichonympha*, and *Holomastigotoides*. They all reveal a similar highly complex flagellar system differing only in minor detail of structure from that found in other flagellates, but somewhat similar in detail to that which was described for certain species of ciliates by Noirot-Thimothée (119, 123). Each flagellum possesses the usual 11 filaments; the two central ones are enclosed in a sheath and show a helical substructure; the nine peripheral ones are double with one of the subfibrils bearing arms that are directed toward the adjacent outer fiber, thus conveying asymmetry to the flagellum. Between the central and peripheral fibers is a third set of smaller or secondary ones; these disappear in the proximal portion of the flagellum. Ensheathing the flagellum is a triple-layered membrane that is continuous with the plasma membrane. The latter, in the proximal region of the flagellum, possesses anchoring granules; these are connected by transverse fibers to both granules on the flagellar

membrane and the outer fiber of the flagellum (77). *Pseudotrichonympha* possesses a transitional fiber, i.e., located in the region between the flagellum proper and the basal body. In this region both the secondary and central fibers terminate, together with the establishment of a relatively short concentric body (77).

The basal body is limited by the outer fibers which are now triple and tilted clockwise, conveying to it an asymmetry which also occurs in the flagellum. The adjacent outer fibers of the basal body are composed of three subfibers linked peripherally by a filament, and each set of fibers is connected to the central one by a different filament. Some organisms have their basal bodies linked together as in *Chlamydomonas* (174).

Reservoir.—The neck of the pear-shaped reservoir of *Euglena gracilis* is lined with a single membrane, below and adjacent to which are parallel groups of longitudinally arranged filaments. Surrounding these are circularly arranged fibers. In its more bulbous portion, filaments have been observed to line the reservoir; others are attached to basal bodies (197). Fibers of a similar nature are present under the reservoir in *Peranema;* possibly these serve to supplement the contractile properties of the protoplasm in this region (155).

Kinetoplast.—The kinetoplast in trypanosomid flagellates is an elongate body surrounded by a double membrane and located just posterior to the blepharoplast (kinetosome). Its ultrastructure resembles that of mitochondria to the extent of possessing transversally oriented double membranes (cristae?) (3, 28, 29, 33, 34, 38, 41, 90, 92, 93, 114, 116, 117, 141, 142, 147, 181, 182). However, unlike mitochondria, the kinetoplast in some forms [*Crithida fasiculata* (38); *Leptomonas* and *Blastocrithidia* (39)], consists of a Feulgen-positive central part and a Feulgen-negative peripheral portion. Stainability is decreased but not abolished by prolonged treatment with deoxyribonuclease. Chakraborty & Das Gupta (29) describe an ultrastructure for the kinetoplast of *Leishmania donovani* as resembling that found by other investigators, but called it parabasal body. It has been pointed out in this review that the parabasal bodies of other flagellates resemble the Golgi complex of higher organisms, and the parabasal apparatus of *Lophomonas blattarum* is unlike that seen in any other form. Perhaps the term "parabasal body" should be deleted when reference is being made to the kinetoplast.

Contractile vacuole.—In trypanosomids the contractile vacuole appears as a clear area closely associated with the Golgi material (34).

Axostyle and axial filament.—In *Tritrichomonas muris* the axostyle is seen as a rod-shaped body whose surface is composed of obliquely arranged fine filaments (4). Near its posterior end is the "chromatic ring," the fine structure of which is composed of a group of granular studded membranes concentrically arranged around the axostyle; they may represent a part of the endoplasmic reticulum. In *Joenia annectens* and *Pyrosonympha vertens* the axostyle is a highly fibrillar structure with filaments oriented mainly in the long axes of the body; it may serve as a contractile structure in these organisms (71).

Costa and paracostal bodies.—The costa in *Trichomonas foetus* has been

described as a series of striations (105) and in *Tritrichomonas muris* as a striated fiber, resembling in some respects the structure of collagen (4, 178). Its slender proximal portion appears to be attached to the base of the kinetosome of the recurrent flagellum (7). The paracostal bodies display double membrane envelopes and an internal structure composed of small granules and vesicles; they may be a form of highly modified mitochondria (4).

Parabasal body and parabasal filament.—The parabasal body does not arise from a kinetosome of the blepharoplast complex. In a variety of flagellates, it displays an ultrastructure comparable to the Golgi material of higher organisms, i.e., composed of profiles of parallel membranes (sacs) and associated smaller vacuoles (4, 12, 13). In *Trichonympha* the parabasal bodies contain acid phosphatase and a quantity of polysaccharide (82). In *Joenia* they appear to divide (73) but are thought to arise *de novo* in certain other forms.

Adjacent and parallel to the parabasal body in some organisms is the parabasal filament. In *Tritrichomonas* and *Trichonympha* it is in association with the basal bodies of the flagella and shows an axial periodicity (2, 4, 82, 136). Some consider it to be a supporting structure (4). The so-called parabasal apparatus in *Lophomonas blattarum* (18) is unrelated to the parabasal apparatus in other forms; its ultrastructure has been discussed under the heading of Nucleus.

Membranes, mitochondria, and Golgi material.—Extending down the central axis of *Lophomonas striata* and *L. blattarum* and enclosing the nucleus is a trumpet-shaped body, the calyx. The anterior surface is covered by a tuft of flagella, and in the region of the basal bodies the calyx membrane appears continuous. Below this region in the vicinity of the nucleus it seems to be composed of a series of longitudinally and obliquely arranged membranous plates; these gather below the nucleus to form a bundle which extends posteriorly as the "axial filament." The calyx, in part, separates the nucleus from the relatively large mass of extracalyx cytoplasm (17, 18).

Some flagellates possess typical mitochondria (with well-defined tubules or cristae); others show less typical ones that are sometimes relatively few in number and possess ill-defined internal structure (4, 197). For example, structures such as the paracostal bodies are of questionable mitochondrial nature (4). In general, except for a few organisms, the mitochondria are less numerous and display a less well-defined structure in flagellates than they do in most groups of protozoa.

The Golgi material, on the other hand, is well developed in most flagellates, displaying the typical arrangement of parallel membranes (sacs) and vacuoles (4). These often make up a large part of the parabasal body and they are said to be in a state of flux, i.e., the number of new sacs or membranes being added to the parabasal body equal in number to those lost through the formation of cytoplasmic vacuoles (82). In some organisms (*Chlamydomonas*), the dictyosome membranes seem to be in direct continuity with the surface membrane of the chloroplast and certain of the larger vacuoles (174).

Most flagellates show some form of endoplasmic reticulum. In *Chlamydomonas*, evidence was found to indicate that the two membrane systems (endoplasmic reticulum and Golgi material) may, at times, show direct continuity. Sometimes, the membranes of the endoplasmic reticulum appear to be in continuity with both the nuclear and chloroplast envelopes (174).

Chloroplast.—Euglena gracilis and *E. viridis* possess membrane-bound chloroplast with an ultrastructure comparable to that in other protista, algae, and certain higher plants (78, 81, 174, 197, 198, 199). These are laminated bodies composed of relatively dense layers of lipid or lipoprotein substance alternating with a less dense layer of aqueous protein substance. The pigment is thought to be present as monolayers at the protein-lipoprotein interface. The pyrenoid appears in or near the middle of the chloroplast where the lamination is interrupted. Of interest is the fact that dark-adapted organisms show a disorganization and partial degeneration in the ultrastructure of the chloroplast; however, this is reversible as evidenced by the fact that animals returned to light have the original ultrastructure of their chloroplast restored (197). Within the chloroplast of *Chlamydomonas* occur the following structures: lamellae, granular matrix, starch grains, pyrenoid body, and eye spot (174).

Pharyngeal rods.—In *Peranema* the two anteriorly connected pharyngeal rods are usually seen lying beside the flagellar reservoir. They are composed of numerous tubular elements surrounded by a denser homogeneous layer. A system of membranes is attached to the rods and extends anteriorly (155). The rods are probably involved in the feeding process of the organism (32).

Ciliophora

Architecturally, the ciliates are equally, if not more, complicated than the flagellates. However, it is becoming increasingly evident, as pointed out above, that the ultramicroscopic structure, especially of the organelles, is much the same in all cells; that is, they all possess a similar molecular pattern which seems to be correlated with a given function.

Generalized cortex and associated fibrillar systems.—The exact nature of the cortex or surface structure of *Paramecium* has been a question of long standing and one of considerable conflict of opinion. It has been claimed that the cortex possesses at least three strata of fibrillar systems: (*a*) an outer polygonal-fibrillar complex; (*b*) an inner fibrillar system which is composed of longitudinally directed fibers interconnecting the basal bodies of the cilia; and (*c*) a third fibrillar system located at the level of or below the basal bodies of the cilia in a plane parallel to the surface of the organism. These fibrillar systems are often highly argentophilic, and have been assigned various functions, such as supportive, contractile, and conductile (144, 153, 154). For references and a detailed discussion of these views set forth in the works of Schuberg, Klein, von Gelei, and Lund, the reader is referred to Taylor (183) and to Witchermann (189); for references to the work of Parducz, see Ehret & Powers (53).

Electron microscopists (52, 53, 54, 110, 111, 112, 176) find essentially the

same layers present in the cortex of *Paramecium* but disagree as to the interpretation of their ultrastructure. The following fine structure of the cortex has been described: (*a*) A double membrane pellicle (outer parabasal membrane). The membranes are interrupted at the position of the cilia and trichocysts; the outer of the pair becomes continuous with the outer membrane of the cilium; the inner one joins with the plasma membrane (inner parabasal membrane). A space (peribasal space) is seen between the pellicle and the plasma membrane in the region between the cilia and the trichocysts. (*b*) The middle or kinetodesmal fiber and associated cilia and kinetosomes, or basal bodies, are often referred to as infraciliature (30, 31, 110). The cilia extend in rows from the depression centers of the surface polygons. Each cilium, designed geometrically like the flagellum, is ensheathed by a membrane which is continuous with the pellicular membrane (165, 190). In *Nyctotherus* and *Euplotes* protrusions exist on this membrane (98, 151, 152). The proximal end of the cilium is continuous with the basal body or kinetosome. The region separating the cilium and the basal body is marked by a transverse plate located at the level of the plasma membrane; it is at this point that the central fibers terminate (120). However, in *Euplotes* as in certain other organisms, they continue into the basal bodies (135, 152). In paramecia about to divide, an accessory basal body is sometimes present; it may have been budded off in some way from the parent [(98, 176); also see (188)]. Metz and associates (110, 111, 112), using the electron microscope to study fragments of whole mounts of the pellicle of *Paramecium* and *Tetrahymena geleii*, found the fibrillar system to consist of a number of parallel units, the kinetics. Each of these is composed of a longitudinal row of basal bodies (kinetosomes), one at the base of each cilium, and a longitudinal ectoplasmic fiber (kinetodesma). The last, which is anteriorly polarized, is composed of smaller overlapping fibrils, each arising from a single basal body. They extend for approximately five or six interciliary units before terminating [also see (30, 31)]. In the marine ciliate *Condylostoma spatiosum*, each kinetodesmal fiber is composed of nine fibrils. Upon joining the lower surface of the fibrillar bundle (kinetodesma), the fibrillae separate to form "a sheet" which extends upward and posteriorly for about 6.5 μ (200). Recently, a large-scale isolation of the kinetosomes from *Tetrahymena pyriformis* has been accomplished (175). Their chemical composition includes 50 per cent protein, 6 per cent carbohydrate, 5 per cent lipid, 3 per cent DNA, and 2 per cent RNA.

The undischarged trichocysts are located between the basal bodies. They possess a "carrot-shaped body" with a dense point that is capped by a material of low density often containing granules. When discharged they display a relatively long striated shaft with a sharp point (21, 59, 96, 100, 115, 138, 163). Related structures (toxicytes) have also been described in certain ciliates (46, 49). An infraciliary lattice system of fine fibrils arranged parallel to the body surface and located in the cortex at the level of the kinetosomes has been described; it probably represents the third fibrillar system of von Gelei (176).

Most authors are in agreement concerning the nature of the kinetodesmal system of *Paramecium;* however, they disagree as to the interpretation of the structure of the outer and inner layers. Ehret & Powers (53) deny that both the outer and the inner layers are fibrillar systems; instead, they have substituted " . . . a corpuscular organelle-packing concept for the 'layered wire' models of cell-border organization," that is, to them the basic unit of surface structure is the ciliary corpuscle, and it is the close packing of these bodies when viewed in cross section that gives rise to the characteristic surface polygon pattern (53, 54). In conclusion, they state that "With the 'fibrillar lattices' eliminated, the old questions regarding their interconnectives seem pointless: the cilium and kinetodesmal fibril appear to be intergral components of a corpuscular organelle that constitutes a primary packing for the complexes and systems of the free cell border" (53). Hence, from this work and that of others, it seems increasingly evident that part, at least, of the silver line system of *Paramecium* is artifactual.

A more complex infraciliature or kinetodesmata occurs in *Stentor* than is present in *Paramecium* (146). However, the structure of the cilia and kinetosomes is essentially the same in both. Beneath the pellicle are found large narrow microfibrils (*KM* fibers) running parallel to the length of the body, one per cortical body stripe. These are made up of stacks of fibrillar sheets each individual fiber of the sheet connecting with the kinetosomes of the basal bodies. Hence, they are considered homologous to the kinetodesmata of Chatton & Lwoff [(30); also see (49)]. Beneath the *KM* fibers in the lower half of the organism and running in the same direction are additional discrete structures termed *M* bands, one to each central stripe. They appear to be laterally joined to each other through side branches and to be in contact with certain vacuolar membranes of the cytoplasm. Fauré-Frémiet, Rouiller & Gauchery (57) show, in the cortex of *Stentor roseoli,* contractile myonema composed of lamina placed side by side, each of them in turn formed by stacks of narrow, thin sheets. The lamina are fixed together by the formation of thicker and denser sheets at their outer edges [also see (152)]. The *KM* fibers and *M* bands suggested by Randall & Jackson (146) are probably comparable to the "myonemes ectoplasmique" and "myonemes endoplasmique" of Fauré-Frémiet & Rouiller (63, 64). In *Spirostomum ambiguum* the myonemes are located in the cortex and consist of about 10 sheets (143, 144, 145). One end of these is attached to the pellicle; the other is unattached. The cortex of *Nyctotherus ovalis* (98) possesses many vacuoles and a tangled cytoplasmic network of fibers. The rootlets from closely spaced cilia often show anastomoses and to be associated with fibrils or hollow tubes which simulate endoplasmic reticulum. Basal bodies with sprouting cilia were sometimes observed. Relatively large and dense granules have been seen in the cortex of *Spirostomum* (65, 66, 145), *Dileptus anser* (49), and *Lacrymaria* (20); their nature and function are unknown.

In addition to the interconnected fibrillar system previously described, Roth (152) has observed in *Euplotes* the following to be connected: mem-

branelles and adjacent membranelles; cirrus and membranelles, cirrus and subpellicular filaments; motorium and subpellicular filaments; cilia and adjacent filaments and the region of the micronucleus with unknown structures. Just inside the gullet edge is seen an area where the fibers converge; this, he identifies as the motorium. The inter-related fibrous systems in *Euplotes* probably possess a neuromotor function (153, 154).

The fine structure of contact areas in conjugating *Tetrahymena pyriformis* shows well-defined tubules suitable for the exchange of cytoplasmic material (55, 56).

Gullet and buccal apparatus.—The gullet and region of ingestion in many ciliates is known to be highly fibrillar, a condition probably associated with the need for increased support and contraction in this region.

In both dividing and non-dividing paramecia, studies have been made of the buccal organelles by means of the phase contrast microscope, classical silver methods (137, 183), and electron microscope (51, 53, 54, 176, 184, 185). The reader is referred to these papers for up-to-date accounts of the structure and literature pertaining to this region. It will suffice to say here that Ehret & Powers (53) find the periodic pattern of the buccal region (peniculum, quadrulus, and ribbed wall) in the longitudinal section to be similar to that of the general body cortex, but with a frequency of repetition three or four times as great.

It is well known that among the holotricha, heterotricha, and hypotricha ciliates, a striking fibrillar system is displayed in the region of the gullet and oral groove. In *Nyctotherus ovalis*, the troughlike oral groove (peristome) extends from near the anterior end down the left border to the indentation, whence it extends transversally deep into the cell as a thick-walled cylindrical tube (98). Specialized cilia, and adoral zone of membranelles, cover the length of the groove and extend deep into the peristomal tube. The cilia making up the membranelles possess well-defined basal bodies from the proximal ends of which extend two non-striated rootlets. These join to form a single rootlet which extends for about 1μ into the ectoplasmic layer to join a network of fibers organized into hexagons with corpuscles at their intersections. The infundibulum of *Campanella umbellaria* is similarly constructed (162). The posterior branch of the ectoplasmic band in *Nyctotherus* (98) is 2 to 3 μ in thickness and is made up of fibrils, some of which have a trichitelike structure as in other ciliates (122, 159). Relatively dense plate-shaped aggregations of fibrils (trichites) which end in the elongate fibrillar network are observed along the lip of the peristome.

The walls of the tubes within the tentacles of *Tokophrya infusionum* show nine longitudinally arranged fibrils, the same number as occurs in the peripheral portion of the cilia [(168); also see (160)]. It is also interesting to note that in this organism, the ingested cytoplasm of the prey is formed into food vacuoles instead of mixing directly with the cytoplasm (167).

Nucleus.—The nucleus of some ciliates is surrounded and suspended by a relatively heavy membrane, the karyophore. Electron micrographs of this structure shows it to be composed of ultramicroscopic fibrils (98, 121). In

Stentor the macronuclear envelope is composed of an outer membrane containing tubular openings, an inner membrane made up of tubular vesicles joined to one another by interconnecting sheets (146). Little of significance in the ultrastructure of the chromatin material has been revealed within the nucleus of ciliates except for *Euplotes* (61), *Stylonychia* (157), and *Tokophrya infusionum* (169, 170). In the macronucleus of *Tokophrya*, especially of old or overfed organisms, are observed units of fine, long, closely packed cylinders of hexagons, each being surrounded by a dense wall. The nature of these bodies is not clear. For interesting cytochemical studies on the nucleus, the reader is referred to Alfert & Goldstein (1), Diller (44), Ehret & Powers (50), and Gall (67).

Kappa.—The killer trait of *Paramecium aurelia* depends upon a refractile cytoplasmic particle known as *Kappa*. This body has been identified in electron micrographs as possessing a double membrane and a variable number of concentrically arranged membranes or lamina arranged about a relatively dense core (45, 87). Certain irregular aggregations of dense material and ill-defined microvilli or tubules are sometimes seen within the *Kappa* particle, simulating the structure of mitochondria. It has been hypothesized that the *Kappa* particle may represent " . . . a mitochondrion infected with an alien particle" (87); it also resembles *Rickettsia* and certain bacteria (45). Certain stocks of *Paramecium aurelia* possess, in addition to the refractile (bright) particles described above, a second smaller and fibrous one.

Contractile vacuole.—In *Tokophrya infusionum* the tubule connecting the pore with the contractile vacuole has a changeable diameter. This is thought to be regulated, in part, by the contraction of numerous fine fibrils located near the tubule. In the vicinity of the contractile vacuole are concentrated many small vesicles, canaliculi of the endoplasmic reticulum, mitochondria, small dense particles, and numerous closely packed vesicles resembling dicytosomes (Golgi bodies). It has been suggested that the latter may be responsible for the secretion and concentration of fluids in the contractile vacuole (172).

In *Paramecium aurelia* and *P. caudatum*, the fine structure of the nephridial plasma (62, 179), that is, the cytoplasm surrounding the feeding canals, is found to be a network of fine branching tubules of about 20 mμ in diameter. These nephridial tubules are connected peripherally directly with branches of the endoplasmic reticulum. Only during diastole of the radial canal do the tubules open into the nephridial canal. Peripheral to the nephridial plasma are bundles of larger tubular elements. The walls of the terminal canal, ampulla, injection canal, and contractile vacuole are lined with an osmiophilic membrane. Contractile, tubularlike fibrillae extend in flattened bundles from the top of the ampulla over the injection canal and contractile vacuole to the excretory canal where they encircle the latter as a spiral envelope. These are discussed in connection with the functioning of the contractile vacuole (179).

The contractile stalk of certain peritrichs consists of two parts: an annulus, which is threaded by many longitudinally arranged, striated filaments

and a central canal which appears to be continuous with the endoplasm of the zooid. Knowledge of the precise nature of the junction between the stalk of the zooid is lacking; however, it seems likely that it might be of a modified ciliary origin (143, 145, 161).

In *Chilodochona* the peduncle is composed of a bundle of protein fibers secreted by intracytoplasmic glandular ampullae; this differs from the method of its production in the peritrichous ciliates (58, 161). In *Vorticella* the contractile elements of the stalk, in addition to possessing subunits, are in close relationship with a well-developed membrane-bound canalicular system (endoplasmic reticulum) (180).

Mitochondria, Golgi material, and endoplasmic reticulum.—The mitochondria in ciliates are relatively numerous and display considerable variation in structure (64, 104, 140, 192, 193, 195, 196). In *Paramecium* they often appear tubular. This is attributable, according to Sedar & Porter (176) and Sedar & Rudzinska (177), to the protrusion of the inner member of the double surface membrane. However, Powers *et al.* (139) deny the existence of a surface membrane and they consider the internal structure as a continuum which is interrupted by numerous canals, or think that it is made up of a tightly packed connection of tubules, the walls of which may fuse to produce a continuum of osmiophilic substance. Sometimes in *Paramecium* the mitochondria display discontinuities of their surface through which the cristae and mitochondrial contents are extruded into the cytoplasm (194). In general, the view that mitochondria are free of surface membranes is, for the most part, discredited (166). In *Paramecium* the mitochondria have been described as originating from the nucleus (139), a phenomenon which, if true, seems more special than general in its occurrence [also see (129)].

The Golgi material is, in general, less abundant than it is in most flagellates. However, it is usually present in its typical form of parallel-arranged lamella and associated vacuoles (118).

Endoplasmic reticulum is present as isolated groups of a few membranes or as an extensive network of tubules (179). Its relationship to the contractile vacuole has been discussed under the heading of contractile vacuole.

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INTERACTIONS BETWEEN PESTICIDES AND SOIL MICROORGANISMS[1,2]

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Although study of interactions between soil microorganisms and the new pesticidal organic chemicals began less than 15 years ago, an extensive literature has by now appeared. Much of this has been published since May, 1958, when reviews by Martin & Pratt (93) on "Fumigants, Fungicides and the Soil," by Eno (34), "Insecticides and the Soil," and by Newman (104) on "Herbicides and the Soil" were featured in the *Journal of Agricultural and Food Chemistry*. Annotated bibliographies on the effects of herbicides and insecticides on soil microflora are available from the Commonwealth Bureau of Soils (20, 21). A review paper by Fletcher (41) on the effect of herbicides on soil microorganisms has appeared in the proceedings of a symposium held at Oxford in 1960.

Except for reference to some of the pioneer investigations, the present review is concerned mainly with publications of the past decade and with outlining the limitations and difficulties encountered in studies of this kind. Ancillary implications are mentioned because of growing concern over the rapidly increasing development and widespread use of highly potent chemicals on the soil for control of weeds, insects, nematodes, and plant pathogenic fungi. Early evidence of such concern was emphasized at the Soil Microbiology Conference held at Purdue University in June, 1954, where a discussion was held on insecticide, fungicide and herbicide effects on microorganisms in soils [Bollen (8)]. This was attended by U. S. Department of Agriculture and University representatives, entomologists, plant pathologists, and soil scientists. Summarized results from several states at that time indicated essentially no evidence of serious injury to soil microorganisms from any of the pesticides in common use. It was recommended, however, that studies be continued with newly developed insecticides and other pesticidal chemicals as they become agriculturally important. That this has been done to a large extent is evident from the present accumulation of literature representing contributions from many countries.

[1] The survey of literature pertaining to this review was concluded in December 1960.

[2] The following abbreviations will be used: BHC (benzene hexachloride [1,2,3,4,5,6-hexachlorocyclohexane]); CIPC (isopropyl-N-(3-chlorophenyl) carbamate); 2,4-D (2,4-dichlorophenoxyacetic acid); DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane); DNOC (4,6-dinitro-2-methyl phenol); EDB (ethylenedibromide); ha (hectare); IPC (isopropyl-N-phenyl carbamate); MCPA (2-methyl-4-chlorophenoxyacetic acid); 2,4-T (2,4-trichlorophenoxyacetic acid); TBA (2,3,6-trichlorobenzoic acid).

Agriculture annually treats millions of acres of soil with millions of pounds of pesticidal chemicals, together with various adjuvants, synergists, carriers, diluents, and solvents. The potency of many of these chemicals is extremely great. Applications of 5 p.p.m. of 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4,-*endo*, *exo*-5,8-dimethanonaphthalene (aldrin) or 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*-*exo*-5,8-dimethanonaphthalene (dieldrin) continue to give protection from potato tuber flea beetle damage after 12 years; 1 to 2.5 p.p.m. are still effective against flea beetles and wire worms after 9 years [Morrison (97)]. This represents concentrations of only 0.5 to 2.5 p.p.m., one acre of mineral soil to a depth of $6\frac{2}{3}$ inches being considered as 2,000,000 lb. on a dry basis.[3] Even lower rates are adequate with some herbicidal chemicals, and many fungicides and fumigants show remarkable degrees of toxicity. In general, on an equivalent molecular basis, many pesticides are characterized by greater potency than are germicides, sulfa drugs, and antibiotics. This is especially true for toxicants such as aldrin, in which the water solubility is less than 0.1 p.p.m.

The increasing number and widespread use of these potent chemicals has aroused concern for the possible injurious effects on soil microorganisms and their activities of significance in soil fertility. Since the pioneer reports of Smith & Wenzel (121) and of Wilson & Choudhri (139) have appeared, many others have studied these effects under laboratory and field conditions. At present the number of chemicals being introduced as pesticides is so great that relatively few even of the most promising can be adequately investigated by soil microbiologists. Moreover, the extreme potency and the insolubility in water of many of the compounds pose experimental difficulties. Because diluents and solvents may produce their own effects, only the active ingredient in its pure state should be used in the laboratory. Crude products may contain isomers or impurities that produce results not attributable to the compound being investigated (139). Treatments with only a few p.p.m. of a water-insoluble substance require mixing a few milligrams with a kilogram of soil; organic solvents cannot be used without producing their own depressive or stimulating influences on at least certain groups of microorganisms. The difficulties encountered in the process of adequate mixing and sampling are obvious, and it is customary to include treatments surpassing normal field rates by a factor of 10 to 100 or more. This has the advantage of forcing effects and of revealing factors of safety. When commercial formulations are studied it should be borne in mind that the mineral carriers of some chlorinated organic insecticides are chemically treated to prevent marked decomposition of the active ingredient during storage. Heptachlor dusts, for example (1,4,5,6,7,8,8-heptachloro-3a,4,6,6a-tetrahydro-4,7-methanoindene), are effectively stabilized by oxygen-containing chemicals such as diethylene glycol. The deactivator additive may be present to the extent of 10 per cent

[3] In this review all rates of treatments given in the original articles as pounds per acre or kilograms per hectare are expressed as p.p.m. in cases where it is clear that the pesticide was mixed with the soil. Otherwise, as with surface applications, the rates given by original authors are used.

[Malina *et al.* (90)]. In some cases, this may have more influence upon microbial activities than does the pesticidal chemical.

In considering the effect of pesticides and other agricultural chemicals applied to the soil, nominal rates of application must be distinguished from ranges that result in practice. A treatment of 50 lb. per acre corresponds to 50 p.p.m. if distribution to a depth of $6\frac{2}{3}$ in. is uniform. Most methods of application, however, result in concentration zones so that lateral and vertical mosaics of varying concentrations result. Strips of 100 p.p.m. or more may shade down to essentially zero concentration between zones where the chemical is actually applied, and tillage operations may result in some lateral transportation. In laboratory studies and often in field plots, treatments are usually made in a manner to obtain a distribution as uniform as possible throughout the soil sample. This is one reason why applications higher than field rates are usually included in the laboratory.

It is not out of place to remind workers with these chemicals to use adquate precautions against the health hazards involved. For information concerning these hazards, consult Frear (44), or the manufacturer.

Laboratory studies on soils under controlled conditions are appropriate but also give results that may not necessarily follow in the field, where changes in environmental factors, drainage, and plant roots introduce variables. Field studies are necessary for practical conclusions and recommendations. Again, because of low rates of treatments, adequate distribution and sampling are serious problems. Fortunately, despite these limitations, research on many of the more important pesticides has been able to show that only when they are applied to the soil at many times the recommended field rates do they appreciably reduce beneficial activities of soil microorganisms.

Pure culture studies and use of artificial media yield desirable information but the results may not always be projected with certainty to the field where the soil as a medium, the environment, and the population as a whole are exceedingly complex and variable.

Consideration must be given also to the effects of microorganisms on the pesticide. Microbial attack in some cases may be essential to activate an herbicide, such as sodium 2,4-dichlorophenoxyethyl sulfate (2,4-DS), which is non-injurious to plants on direct contact. *Bacillus cereus* var. *mycoides* hydrolyzes this to 2-(2,4-dichlorophenoxy) ethanol, which is then oxidized to 2,4-D [Vlitos (130)]. Acids also effect the hydrolysis, and such action by microorganisms has been attributed to acids secreted during their metabolism. On the other hand, microbes may inactivate or destroy the pesticidal properties of a compound. Substituted urea herbicides, especially, are susceptible to relatively rapid microbial decomposition [Abel (1); Hill *et al.* (56)]. Many *Pseudomonas* species attack these as well as other kinds of weed killers (32). With many herbicides this is desirable because it permits subsequent seeding and growth of susceptible crop plants with minimum delay. Such is the case with calcium cyanamid, which can serve first as a weed killer and subsequently as a nitrogenous fertilizer, although the primary decomposition may be catalytic [Crowther & Richardson (26); McCool (84)]. The rapid

decomposition, at least in part microbial, of the highly toxic phosphate insecticides such as O,O-diethyl-O-*p*-nitrophenyl thiophosphate (parathion) is desirable from the standpoint of public health. This is true for pesticides in general. Some are now beginning to appear in water supplies, and concern is developing regarding possible effects on man and on animal life, both domestic and wild. Although part of this contamination results from treatment of lakes, streams, and irrigation ditches to control trash fish and weeds, at least some could be the result of leaching from the soil [Ogle & Warren (108)]. It therefore behooves not only soil microbiologists but all soil scientists as well, to study the fate of pesticides in the soil. How organic and inorganic colloids, ion exchange complexes, organic matter, and other physical and chemical features of the soil influence adsorption, fixation, activation, inactivation, persistence, transformation, leaching, and volatilization of chemicals applied for the control of weeds, insects, fungi, and other pests, are important questions remaining largely unanswered. Because differences often occur in pest control with a given pesticide on different soils, the studies must embrace a considerable variety of soil types representing a broad range of pedological characteristics. The differences in such characteristics and their influence on microbial activities in soils following minute additions of potassium gibberellate, has been recently reported by Chandra & Bollen (16). Lichtenstein *et al.* (80) showed that climate and organic matter were the major factors affecting the persistence of aldrin, DDT, and lindane, the *gamma* isomer of benzene hexachloride (BHC), in soils from Kansas, Ohio, and Wisconsin. Aspects of soil chemistry influencing the behavior of pesticides in soil have been outlined by Dean (28). Freed (43) has considered physico-chemical relationships between herbicidal chemicals and their environment under field conditions.

Fungicides and Fumigants

Fumigants are chemicals that form vapors or gases which destroy insects and other pests. Many are especially effective against fungi and they are therefore included with the fungicides in this discussion.

Domsch (30), studying the effects of several soil fungicides at doses of 60 to 400 p.p.m. found that commonly occurring bacteria, actinomycetes, fungi, and unicellular algae were affected differently. Sodium N-methyldithiocarbamate (Vapam) increased the development of actinomycetes but decreased the growth of the other microbes. N-trichloro-methylthiotetrahydrophthalimide (Captan) inhibited all but the bacteria, while disodium ethylene bisdithiocarbamate (Nabam) inhibited fungi only. Allyl alcohol, important as a herbicide against dormant seed, inhibited the growth of algae and fungi and increased the numbers of bacteria. Jensen (65) isolated strains of *Pseudomonas fluorescens*, *P. putida*, *Azotobacter*, *Nocardia corallina*, and *Trichoderma viride* and found that they were able to utilize this alcohol.

Martin *et al.* (92) found, after several sandy loam soils had been fumigated with CS_2, chloropicrin, D-D (mixture of 1,3-dichloropropene, 1,2-dichloropropane, and related compounds), ethylene dibromide, and vapam,

that a marked reduction of the fungus population occurred. The fungi became reestablished, but represented fewer species in comparison with those in control soils; in this respect, the populations were still markedly affected even after three years. *T. viride* was stimulated by the fumigation in many cases.

The selective influence of different fungicides on the microbial population of the soil is illustrated by experiments of Corden & Young (23). The fungal population of a sandy loam soil decreased continually during the first month following treatment with 3,5-dimethyl-1,3,5-2H-tetrahydrothiadiazine-2-thione (mylone) at 30 p.p.m., but the soil could be easily reinfested with the normal fungal flora during this period. Although fungi were greatly reduced, the total bacterial population increased while numbers of actinomycetes remained unchanged. Nabam at 20 p.p.m. had similar effects. However, the total fungi eventually increased solely as the result of development of *Trichoderma*. Vapam, at 15 p.p.m., caused a general decrease in total fungi; certain penicillia contributed to a build-up during the third and fourth weeks. Numbers of bacteria and actinomycetes were unaffected by this fumigant. In soil treated with 20 p.p.m. methylmercury oxinate, the total fungi decreased more than 95 per cent within one day; then owing primarily to the development of species of *Penicillium*, built up rapidly to three times the original numbers. The oxinate did not change bacterial counts during four weeks but greatly reduced actinomycetes. 2-Chloro-3-(tolylsulfonyl) propionitrile (CP 30249), at 15 p.p.m., caused a rapid decline in the total number of fungi. The effect was similar to that of vapam but was more pronounced, and the build-up following after one week was again attributed solely to an increase in the numbers of *Trichoderma*. The bacterial population was little affected by the nitrile except for an increase in a species insensitive to streptomycin. Actinomycetes were slightly reduced.

Trichoderma viride is often prominent in the fungal population developing after soil fumigation and this fungus, rather than the direct fungicidal action of applied chemicals, may destroy certain pathogens such as *Armillaria mellea*. The frequency with which *T. viride* has been reported as a dominant recolonizer in fumigated soils appears to result as much from its high growth rate as from its tolerance (117).

Bollen *et al.* (11) noted that D-D decreased bacteria as well as molds, and that the effect persisted in stored air-dry samples for 10 months.

Certain fungi can become adapted to fungicides. Strains of *Botrytis cinerea*, resistant to 250,000 p.p.m. captan suspended in agar media, were developed by Parry & Wood (109). Tetramethyl thiuram disulfide (thiram) was toxic above 31 p.p.m. and ferric dimethyl dithiocarbamate (ferbam) above 125 p.p.m. Nabam, zinc ethylene bisdithiocarbamate (zineb), and zinc dimethyl dithiocarbamate (ziram) prevented growth at similarly low concentrations. An adapted strain resistant to 5,000 p.p.m. ferbam was as susceptible as the parent to nabam, thiram, zineb, and ziram. *Venturia inaequalis* could not be adapted to any of these fungicides.

A study of various fumigants, fungicides, and bactericides by Moje *et al.*

(95) revealed that toxicity to soil organisms and growth of orange seedlings in an old citrus soil is associated with halides and alcohols containing *alpha-beta* unsaturation. Certain chemicals, notably sorbic and acetylenedicarboxylic acids, greatly stimulated the development of *Trichoderma viride*. Because *T. viride* is known to be antagonistic to *Phytophthora*, *Pythium*, and *Rhizoctonia* these results suggest the possibility of indirect biological control of such pathogenic fungi. The relationships between unsaturation, substituents, and other aspects of chemical structure of organic compounds to their toxicity against bacteria, fungi, and nematodes have been discussed by Moje (96).

Bollen & Lu (10) made plate counts on composite samples taken 10 days after the fumigation of replicate plots on Chehalis silty clay loam soil. Ethylenedibromide, methyl bromide, dichloropropenes (telone), and vapam increased bacterial counts but lowered the percentage of *Streptomyces*. 1,2-Dibromo-3-chloropropene, with 33 per cent other halogenated C^3 compounds (nemagon), was weakly depressive. Mold counts were slightly increased, except by telone and vapam. The increases could be attributed to promoted activity of the surviving population, a phenomenon that often follows any partial sterilization of the soil. In laboratory studies by Roa (114), the same fumigants were used at field rates and also in concentrations 10 times greater. Carbon dioxide evolution from the decomposition of native organic matter was increased except by vapam, which also retarded decomposition of added dextrose. All compounds slightly increased ammonia production from the native organic matter but temporarily depressed the ammonification of added peptone. Nitrification was retarded for a few weeks in all cases. These results are similar to those found by Munnecke & Ferguson (100) for the influence of vapam, methyl bromide, and chloropicrin on ammonification and nitrification, and to those of Jones (70) who noted that EDB over a wide range of concentrations stimulated ammonification. No significant effects were observed of any of the fumigants on oxidation of sulfur to sulfate. Pure culture studies showed that *Bacillus subtilis*, *Escherichia coli*, *Streptomyces griseus*, and *Penicillium chrysogenum* could, after a few days induction, grow well with vapam as the sole carbon source.

Wolcott *et al.* (140) found that telone treatments caused a seven- to eight-week lag period in nitrification, and pointed out the significance of this in altering seasonal distribution of ammonium and nitrate nitrogen in the field.

The effects of mylone and nabam on microbial numbers, nitrification, and carbon dioxide evolution in four widely different soil types were studied by Chandra & Bollen (17). Because air-drying may influence subsequent activities in incubated soils, samples were used immediately as obtained fresh from the field after passing through a 10-mesh sieve. The moisture content approximated one-third of field capacity in each case. The fungicides were used at field rates: mylone (85 per cent active ingredient) at 300 lb. per acre, and nabam (19 per cent active ingredient) at 200 lb. per acre. Plate counts

showed a depression of bacteria for 30 days, with little change in percentage of *Streptomyces*, while molds were depressed for 45 days. Nitrification of ammonium sulfate and of ammonium hydroxide added with the fungicides was completely inhibited for 30 days; when added 30 days after the fungicides, nitrification of ammonium hydroxide was depressed only 50 per cent at 30 days. Soil respiration was retarded for 28 days, the effect lessened at 42 days, while, at 56 days, the treated soils evolved more CO_2 than did the control. Significant differences at the one per cent level were obtained for soils, fungicides, and interactions. In all of these effects mylone was slightly more potent.

The effectiveness of a fungicide used on the soil may depend largely on the method of application. Assays by Corden & Young (22) on a sandy loam soil infested with *Fusarium oxysporum* f. *cubense* showed that ED_{50} for nabam, mixed with the soil was 6 p.p.m.; as a drench, $ED_{50} = 600$ p.p.m. for a depth of 5 to 6 cm., most of the chemical being absorbed at the soil surface.

The fungicides 98 per cent tetrachloro-*p*-benzoquinone (spergon), 50 per cent 2,3-dichloro-1,4-napthoquinone (phygon), and 7.7 per cent ethylmercury *p*-toluene sulfonanilide (ceresan M), used extensively for seed treatment, have varied toxic effects on different species of *Rhizobium*. Experiments by Hofer (58) showed that the bacteria for alfalfa were injured least while those for clover were the most sensitive. Ceresan M was more toxic than the other two chemicals, a dilution of 0.5 p.p.m. being critical for growth of the clover bacteria on Szybalski (125) gradient plates. These results indicate the advisability of testing fungicides proposed for use on leguminous seeds for their interaction with inoculants. Ferbam, ziram, and zineb in soil at 50 p.p.m. are temporarily toxic to nitrification (138).

A water-miscible, urea-formaldehyde concentrate containing 85 per cent active solids combined in a urea to formaldehyde mol ratio of approximately 1:4.6 (UF-85), is effective in controlling *Streptomyces scabies*, the pathogen causing common potato scab [Bartz & Berger (5)]. It also shows promise for symphylid control (98). The formulation behaves like a mixture of methylureas and formaldehyde when diluted with water, and its reactions in the soil may be expected to be typical of these chemicals. The concentrate, diluted with water, is effective for potato scab at 150 gallons per acre and for symphylids (98), at 30 gallons. This adds, in water-soluble form, 192 and 38 lb. of nitrogen, respectively; in contrast to the nitrogen in urea-formaldehyde resin fertilizers (Urea-Forms), it should become readily available plant food.

Chlorinated hydrocarbon insecticides are fungitoxic in proportion to their water solubility and vapor pressure (113). Tested by plate culture technique against *Rhizoctonia solani*, lindane, which has a relatively high water solubility (7.3 p.p.m. at 25°C.), was most toxic in supersaturation at 25 p.p.m. Aldrin, dieldrin, 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*, *endo*-5,8-dimethanonaphthalene (endrin), and 1,1,1-trichloro-2,2-bis(*p*-anisyl)ethane (methoxychlor), having solubilities of a few tenths p.p.m., had a low fungal toxicity. Inhibition of mycelial growth fol-

lowing the exposure of fungus inoculum to vapors of the insecticides was proportional to their vapor pressures. Heptachlor was most effective; DDT had no effect.

The sensitivity of certain fungi to mercury compounds, thiram, and other pesticides used in seed treating for control of insects and plant diseases, provides a bioassay method to determine whether or not seed has been treated in conformity with label statements. Treated seeds placed on an agar medium inoculated with spores of the fungus will, after incubation, show a surrounding mycelium-free zone, the size of which is indicative of the dosage of the pesticide [Crosier (25)]. Munnecke (99) used agar media seeded with *Myrothecium verrucaria* for the bioassay of non-volatile, diffusible fungicides. Plugs of treated soil were placed on poured plates and the surrounding clear zones measured at 48 hr.

Enzymatic fungicides that attack the fungal cell wall show promise in plant disease control. Farr & Hilborn (36) found that *Aspergillus niger* spores treated with 0.1 p.p.m. chitinase in a preparation from *Calvatia gigantea* were destroyed by dissolution of the cell wall and exposure of the protoplast. Their findings suggest that this approach may be extended to include enzymes that specifically attack other constituents of cell walls, and research may develop applications for the control of insects and other organisms.

Certain kinds of organic matter, such as soybean hay, added to the soil under proper conditions may be indirectly fungicidal. Davey & Papavizas (27) have shown that *Rhizoctonia* can be so suppressed, evidently as a result of increasing numbers of antagonistic *Streptomyces*. The fungicidal properties of sphagnum moss are well known to horticulturists. Seedlings started in this are rarely attacked by damping-off fungi (24).

The use of certain fumigants and herbicides to retard biological decomposition and consequent subsidence of muck soils has been suggested. However, Stotzky *et al.* (124) showed that CIPC, CRAG Fungicide 974 (mylone), and ethylene dibromide and several other fumigants were not effective on Rifle peat. CIPC actually enhanced the decomposition.

McCalla *et al.* (83) suggested artificial inoculation with an organism such as *Stachybotrys atra* as an aggregator following soil fumigation to combat plant pathogens. Such aggregators have little influence on soil structure unless the natural microflora is greatly reduced.

Burgess (14) presents a resume of comparative microbial effects resulting from soil sterilization with steam and with fumigants such as formaldehyde and carbon disulfide. An extensive discussion of partial sterilization of the soil by chemicals, and their effects on microbial activities and soil fertility has been given by Waksman & Starkey (131).

Herbicides

Of all the various pesticides that find their way into the soil, organic herbicides appear to be the most susceptible to microbial attack. In large measure, this accounts for the considerable attention that has been given to

their decomposition and duration of toxicity. A discussion of microbial and other factors that alter herbicides in soil has been presented by Sheets & Danielson (120).

Many of the new herbicides are selective and may be applied differently for different purposes. Some are used as foliar or ground sprays. In fallow programs also, whether they be clean-cultivated or stubble-mulch, chemical treatment is important; prevention of weed growth conserves moisture. In whatever manner they are used, sooner or later herbicides find their way into the soil. Incorporation in the soil gives some herbicides greatly increased activity possibly because of some microbial action, and variability in results is brought about by differences in soil moisture. Rates of application as "chemical hoes" for weed control range from less than 1 lb. per acre to 15 to 25 lb.; 2 to 4 lb. per acre is typical for many of the newer compounds. Except where heavier applications or other types of chemicals may be used as permanent soil sterilants to maintain plant-free areas in agriculture, industry, and public works, it is necessary that the toxicant is not injurious to a desired crop, or that it be detoxified before subsequent seedings. In this detoxification, microorganisms often are known to play an important role. On the other hand, herbicidal compounds affect microorganisms, exerting stimulating or depressive effects depending upon the kind of chemical, its concentration, and the environmental conditions. For many recently introduced chemicals, the interactions with microorganisms have not been fully investigated.

Although the disappearance of herbicides from the soil is, in many cases, largely attributable to microbial activity, other contributing factors are known. Among these, temperature, pH, and clay components are important. Burschel & Freed (15) concluded that the rate of breakdown of IPC, CIPC, and 3-amino-1,2,4-triazole (amitrol) in soil proceeded as a first-order reaction. On this basis, they calculated the heat of activation required and postulated that such information could be used to estimate the residual life of various herbicides in the soil under field conditions.

Dewey & Pfeiffer (29) reported that the biological decomposition of TBA appears to depend upon the rate of application and the microbial activity of the soil.

Maleic hydrazide, a true growth inhibitor or antiauxin, in concentration used as a herbistat, appeared inactive against *Azotobacter*, *Rhizobium*, and other bacteria tested by Nickell & English (106). Levi & Crafts (78) found that inactivation of this chemical in the soil under warm, moist conditions was fairly rapid and resulted in increased nitrogen fertility. Whether the decomposition was the result of hydrolysis or of microbial action was not determined. The toxicity and inactivation varied in different soils but could not be related to soil characteristics among the 11 soils studied. Toxicity was highest and inactivation slowest in Arbuckle clay loam; 15 p.p.m. were toxic originally, and 340 p.p.m. produced complete sterility that was present five months later. Sterility was obtained with 140 p.p.m. in four soils, but in the

lateritic Aiken clay loam, 680 p.p.m. were not toxic after three months. In soils where anion exchange is predominant as in Aiken clay loam, maleic hydrazide was held by the clay component; in the other soils it moved freely, although relatively large amounts of water were required for downward leaching. Such variation in results with different soils emphasizes the need for investigating the relation of fundamental soil properties to the behavior of applied pesticides. The importance of soil type, clay content, and pH and their effect upon the toxicity of certain other herbicides in some California soils has been reported (53, 54, 79).

Raud *et al.* (111) found 1 p.p.m. of 3-(*p*-chlorophenyl)-1,1-dimethylurea (monuron) to be toxic to *Stichococcus bacillaris* in liquid media; they suggest that the algostatic properties of herbicides could secondarily influence the carbon/nitrogen ratio of soil.

Enrichment cultures from soil used in detoxication experiments with DNOC led to the isolation of *Corynebacterium simplex* by Gunderson & Jensen (51). This organism could use the herbicide as a sole source of carbon and nitrogen when the concentration in a synthetic liquid medium at pH levels between 7.3 and 8.5 did not exceed 0.05 per cent; at 0.5 per cent the compound was bactericidal. The toxicity of DNOC and related compounds is determined by pK and pH, the undissociated molecule being the toxic agent. A *Corynebacterium* that actively decomposed 2,4-D was isolated by Rogoff & Reid (116) from soil percolated with the herbicide at 500 p.p.m. The chlorine was quantitatively released.

By use of the soil percolation technique, Audus (3) showed that detoxication of 2,4-D is attributable almost entirely to microorganisms. A bacterium, probably an *Arthrobacter*, able to use 2,4-D as a sole carbon source, was isolated from the percolate (4). Under field conditions, 2,4-D disappeared from a silty clay loam soil more rapidly than did 2,4,5-T [Newman *et al.* (105)]. A population of organisms capable of rapidly decomposing 2,4-D was built up as a result of treatment with this herbicide, but apparently this was not the case with 2,4,5-T.

Lenhard (77) found that 100 to 1000 p.p.m. of 2,4-D decreased hydrogenase activity as well as the microflora of a South African soil. Rates above 500 p.p.m. caused autolysis of the bacteria and decreased nitrogen fixation by *Azotobacter*.

Magee & Colmer (88), using the Warburg technique, determined that active cells of *A. chroococcum* and *A. agile* were more resistant to the harmful effects of 3000 p.p.m. of 2,4-D triethanolamine salt than were resting cells. *A. agile* retained viability after 2 hr. exposure to 15,000 p.p.m., although respiration was completely inhibited by 10,000 p.p.m. *A. vinelandi* was most resistant, and *A. chroococcum* least resistant to trichloroacetic acid (84). Johnson & Colmer (68) observed that the growth of *Bacillus cereus* in nutrient broth was checked by 500 p.p.m. of 2,4-D, germination of spores was prevented by 300 p.p.m., and ammonification of gelatin was inhibited by 500 p.p.m. With *Pseudomonas fluorescens*, concentrations of less than 5000 p.p.m

had little effect (69). Gelatin ammonification of a soil suspension by microorganisms required 20,000 p.p.m. 2,4-D for complete inhibition. These concentrations greatly exceeded the levels used in field treatments.

By measuring the oxygen uptake in Warburg respirometers containing silt loam soil treated with different herbicides at rates corresponding to those used for weed control, Whiteside & Alexander (136) found no effects exerted by amitrole, 2,4,5-T, or 2,4-D and corresponding propionic and butyric analogues on microbial decomposition of native or added organic matter. Changes in ultraviolet absorption of herbicide solutions inoculated with soil showed that 2,4-D and 4-(2,4-dichlorophenoxy)butyric acid were metabolized by the soil microflora; no decrease in absorption occurred with 2,4,5-T and several other compounds.

Hale *et al.* (52), by soil percolation methods and Warburg respiration measurements, found that nitrification was inhibited by excessive concentrations, 40 to 160 p.p.m., of CIPC and monuron. Such inhibitory concentrations would not occur at usual field applications and there would be little or no detrimental effect on soil nitrification.

Kratochvil (75), by measuring the pressure of gas evolved from a silt loam soil treated with herbicides at near field rates, concluded that TCA and IPC significantly reduced microbial activity whereas 2,4-D and 2,4,5-T were without effect. Sodium 2,4-dichlorophenyl "Cellosolve" sulfate had a stimulating effect.

Virag (129) found dichlorophenoxy- and chlorophenoxyacetic acid and MCPA at 1000 to 4000 p.p.m. depressed the growth of *Aspergillus niger* and several other soil fungi, *in vitro*. Although heavier rates are used in foliar sprays, quantities that reach the soil immediately would be small and unlikely to harm microorganisms.

Fernandes (37) found that nitrification of ammonium sulfate in a Winogradsky medium inoculated with a soil suspension was not inhibited by 2 to 18 p.p.m. of 2,4-D or 10 to 60 p.p.m. of TCA. Fleig (39) found that 2,4-D was inactivated within a few weeks in soils under conditions favorable for microorganisms, and that ammonification, nitrification, and nitrogen-fixation were unaffected. Soil respiration was temporarily reduced in a light soil. In heavy soils these processes were not severely checked by applications below 100 kg./ha. In solution cultures, nitrification was severely retarded by 3 p.p.m. of 2,4-D, but the addition of soil counteracted this effect.

The duration of the activity of fatty acid-type herbicides in soil is economically important; because they are selective, persistence can be valuable for specific crops, but toxicity to succeeding crops may make a rapid disappearance desirable. For this reason, their decomposition has received considerable study. The detoxification is largely or entirely biological because the active compounds remain for long periods in sterile soil.

Data obtained by Holstun & Loomis (59) indicated that the decomposition of 2,2-dichloropropionic acid in soil was primarily microbial. Other factors acting indirectly by affecting activity of the microorganisms were

found to be moisture, temperature, and soil type. Low moisture, low pH, temperatures below 20°C., and large additions of organic matter inhibited the destruction of the herbicide. Thiegs (126) observed that sodium 2,2-dichloropropionate (dalapon) at 50 p.p.m. in soils of different texture decomposed more rapidly as moisture and temperature were increased. After an initial treatment, subsequent applications were decomposed more rapidly, indicating microbial adaptation or an increased population of active strains. The influence of temperature, moisture, and soil texture on detoxification of TCA was studied by Loustalot & Ferrer (82); inactivation was more rapid at warmer temperature, higher moisture, and in sandy soil. Isolates of *Agrobacterium* and *Pseudomonas* found to be active on dalapon were obtained by Magee & Colmer (89). Hirsch & Alexander (57), using liquid media inoculated with soils previously incubated with 250 to 500 p.p.m. of chlorinated pesticides, isolated and characterized strains of *Pseudomonas* and *Nocardia* that actively degraded 2,2-dichloropropionic acid. *Beta*-substituted propionates were more slowly attacked than were corresponding *alpha*-substituted compounds.

Using selective media, three groups of bacteria capable of decomposing chloro-substituted aliphatic acids were isolated from soil by Jensen (63). The organisms were more active in soil than *in vitro*. *Trichoderma viride*, *Clonostachys* sp., and *Acrostalagmus* sp. were able to decompose monochloroacetate, dichloroacetate, and *alpha*-monochloropropionate, in a decreasing order of velocity, with liberation of chloride ions (64). With *T. viride*, the release of chloride was shown to be caused by an adaptive enzyme. Jensen (66) later found that *Pseudomonas dehalogenans*, *Arthrobacter* sp., and *Agrobacterium* sp. produced dehalogenases for these compounds as well as for *alpha*-dichloropropionate.

Biological decomposition of mono- and trichloroacetate and *alpha*-dichloropropionate in soils was investigated by Jensen (67). Monochloroacetate was most readily decomposed. *Pseudomonas* sp. and various fungi, especially *Trichoderma viride* were involved. Trichloroacetate and the propionate were slowly attacked by special groups of bacteria such as *Arthrobacter* and *Agrobacterium*. Bacterial multiplication ensued with the decomposition. The herbicidal effects of trichloroacetate and *alpha*-dichloropropionate disappeared as their chlorine was released as Cl^-. Carbon dioxide production and ionization of the organically bound chlorine showed that the three compounds were decomposed after a variable latency period which could be eliminated by adding active bacterial suspensions.

Elkan & Moore (33) observed that although sodium propionate applied to greenhouse soil at 50 and 100 p.p.m. markedly depressed fungi, the plate counts of total microbes were considerably increased.

By selective enrichment through successive dosing of a soil with 2,4-D, Bell (6) succeeded in isolating a new species of *Achromobacter* which used this herbicide as the principal carbon source and rapidly decomposed it to small

molecules, releasing virtually all of the 2,4-D chlorine as chloride. The bacterium has an apparently adaptive system capable of oxidizing 2,4-D, but can oxidize a variety of analogues and 2,4-dichlorophenol without prior adaptation. Pathways for the degradation have been proposed (7).

Walker & Steenson (132) observed that after repeated sprayings of turf with 2,4-D or MCPA, the soil microflora became adapted to decompose these herbicides; the adapted organisms persisted in the soil for 12 months after sprayings were discontinued. Adapted organisms could also be developed by growth on peptone agar containing the herbicide, which thus need not be the main source of carbon. Species of *Achromobacter*, *Bacterium*, and *Pseudomonas* able to decompose 2,4-D were isolated. Cells from young cultures were more active than older ones in oxidizing 2,4-D and MCPA, and the dissimilation took place through chlorophenolic compounds (122). Steenson & Walker (123) found that *Achromobacter* grown on agar containing either 2,4-dichlorophenol or 5-chloro-2-cresol became adapted to oxidize both 2,4-D and MCPA. A mutant bacterium isolated by Walker & Newman (133) and tentatively identified as a species of *Mycoplana*, was found to decompose 2,4-D rapidly in soil and in synthetic media. Resting cells metabolized 2,4-D completely.

On Winogradsky soil plaques, Colmer (19) observed that the triethanolamine salt of 2,4-D (40 per cent acid equivalent) at 100 p.p.m. stimulated growth of *Azotobacter chroococcum* in a nitrogen-free medium, but 200 p.p.m. were inhibitory. *A. agile*, on the other hand, was stimulated at the 1000 p.p.m. level. This suggested that the two species might be separated on the basis of their differential response. The tolerance of each organism could be increased by serially subculturing in increasing concentrations of the herbicide.

Concentrated activated diesel emulsion (CADE) (67 per cent diesel oil and 33 per cent pentachloro-phenate), 2,4-D, and combinations applied at usual field rates to sandy loam and silt loam soils did not appreciably reduce the nitrate accumulation under laboratory conditions in studies made by Koike & Gainey (74). Higher concentrations in amounts to 50 lb. of 2,4-D and 5000 gallons of the diesel emulsion, inhibited nitrification for 8 to 16 weeks. 2,4-Dichlorophenoxyacetic acid at 25 and 50 lb., CADE at 500 to 5000 gallons, and combinations of the two at 1000 gallons per acre greatly increased the total plate counts of bacteria. Such stimulation of bacterial numbers is not surprising; numerous kinds of soil bacteria, especially *Pseudomonas*, are readily adapted to the utilization of paraffins and other hydrocarbons.

Klyuchnikov & Petrova (73) examined oak rhizosphere samples from a chernozem soil after it had received seven field applications of herbicides during a two-year period, the cumulative treatments amounting to approximately 7 lb. of 2,4-D and 100 lb. of kerosene per acre. Numbers of bacteria were little affected, but actinomycetes were decreased about 50 per cent.

Total molds were increased; *Trichoderma*, which predominated in control samples, were decreased by the herbicide treatment, resulting in a predominance of *Penicillium*.

Fernley & Evans (38) isolated *alpha*-chloro-muconic acid as an intermediate produced by a *Pseudomonas* in the process of metabolizing 2,4-D.

Colonial morphology of certain fungi developing on agar media may be altered by 2,4-D and some related phenoxy compounds; these chemicals at 50 to 3000 p.p.m. caused colonies of *Gloeosporium olivarum* to lose their generally circular outline and become wavy and irregular at the margin (101).

Hill *et al.* (56) measured the rate of disappearance of monuron (Karmex W) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Karmex DL) in light textured soils under field conditions and concluded that microbes play a definite part in the primary decomposition. Some photochemical decomposition can occur when little rainfall follows surface application. Phytotoxic concentrations following treatments of 1 to 2 lb. per acre disappeared within four to eight months. Laboratory studies indicated that even higher concentrations of these herbicides underwent rapid microbial decomposition in incubated soils, and it was shown that *Pseudomonas* sp. isolated therefrom could utilize monuron as a sole carbon source.

Gamble *et al.* (45) found by respiration rates as well as by plate counts that the heterotrophic microflora of a silt loam soil was depressed for one and three months after field treatments with monuron, IPC, and several other herbicides. Inihbition of nitrification by dalapon, TCA, monuron, and several other herbicides was reported by Otten *et al.* (107).

Reid & Duros (112), using a modified soil percolation apparatus and enrichment cultivation, isolated strains of *Corynebacterium* and *Streptomyces* which could destroy aryloxy and chlorinated acid herbicides. Dinitro and substituted urea compounds were attacked by strains of *Pseudomonas*. Pigment production by this genus appeared to be related to the prolonged depression of nitrification which may follow application of dinitros and substituted ureas. Douros (31) found that dalapon, monuron, DNOC, and other related herbicides could be used as energy sources by soil microorganisms. Chlorine substituents favored attack by Gram-positive organisms, especially *Corynebacterium*. Carbamyl, cyano, or nitro groups favored attack by *Pseudomonas* and other Gram-negative bacteria. Some *Pseudomonas* species could utilize dinitro compounds but not urea or substituted ureas; other species could attack the ureas, and still others, both types of compounds. With adequate aeration, *P. desmolyticum* could convert urea quantitatively to nitrate, apparently not via ammonia. At 25 p.p.m., dalapon depressed nitrification in soil for one month, nitro compounds for two months, and monuron for three months. Second applications were depressive for periods of less than three weeks.

The effect of 5 and 100 p.p.m. of 2,3,6-trichlorobenzoic acid, ethyl N,N-di-*n*-propylthiolcarbamate, 3,-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron), and 2-chloro-4,6-bis(ethylamino)-s-triazine (simazine) on respiration in nine

widely different soils was reported by Chandra *et al.* (18). In most cases, the herbicides decreased CO_2 evolution for at least 28 days, after which the inhibition decreased considerably at the end of 56 days. Treatments of 100 p.p.m. were little more depressive than were 5 p.p.m. Although the different soils responded differently, pointing to a significant influence of soil type, there was no correlation with percentage of organic matter, clay content, or cation exchange capacity.

Pochon *et al.* (110) investigated microbial interactions between simazine and other aminotriazine herbicides. Various soils in pots were treated with the chemicals at field rates. Bacteriological analyses made three weeks later revealed no differences between controls and treated samples, except for a slight stimulation of *Azotobacter* and inhibition of *Cytophaga* and *Cellvibrio*. Ammonifiers, nitrifiers, denitrifiers, and amylolytic bacteria were not affected. Carbon dioxide evolution and the enumeration of the total microflora showed no significant effects produced by the herbicides. A heavy dosage of 250 kg./ha. induced no pronounced differences. They pointed out that under field conditions, indirect effects, induced by injuring or killing of plants, could result from the reduction of rhizosphere activities.

The ammonium of ammonium thiocyanate, sometimes used as a weed killer, at 750 p.p.m. is rapidly nitrified and bacteria isolated from such treated soil have been shown to be capable of utilizing the compound as a sole source of nitrogen (118). When applied to soil at this rate, sodium thiocyanate as well as ammonium thiocyanate, increased the numbers of bacteria; 12,500 p.p.m. were only temporarily bacteriostatic and had little effect on algae or protozoa. An autotrophic bacterium, *Thiobacillus thiocyanoxidans* (141), can utilize potassium thiocyanate as a source of energy, carbon, and nitrogen. Sodium cyanide, on the other hand, appears comparatively toxic to bacteria. McCool (85) observed that although 25 p.p.m. sodium cyanide increased numbers of bacteria and *Streptomyces* in soil, they were reduced by 100 p.p.m. and completely suppressed by 200 p.p.m. Mold counts were increased by additions of 125 to 500 p.p.m. of sodium cyanide.

Insecticides

Results obtained by Smith & Wenzel (121), Wilson & Choudhri (139), Bollen *et al.* (11), Fletcher & Bollen (40), and by other early workers in this field indicated that, although certain microbial activities in the soil are disturbed by extraordinarily large applications of insecticides, the effects from normal field rates are not sufficiently great as to result in a significant reduction in soil fertility.

Chlorinated hydrocarbons.—Studies by Bollen *et al.* (11) on samples from field plots freshly treated with various insecticides, revealed that benzene hexachloride (BHC) (1,2,3,4,5,6-hexachlorocyclohexane) and DDT depressed mold counts in several soil types; the numbers of bacteria were not significantly influenced. Laboratory studies on a clay adobe soil with different isomers of BHC at 1000 p.p.m. showed that the *gamma* isomer (lindane) was

more effective in producing quantitative and qualitative microbial responses.

Fletcher & Bollen (40) studied the effect of aldrin at 200 and 1000 p.p.m. in 10 Oregon soils under laboratory conditions. In all cases the aldrin treatments increased the numbers of bacteria, and to a greater extent with added peptone. Molds and *Streptomyces* counts showed stimulations and decreases not consistent with treatment or soil type. The development of *Azotobacter* on soil plaques was not influenced. Only insignificant effects on ammonification, nitrification, and CO_2 production in peptone-amended soils were observed. Roberts & Bollen (115), using a microplating method, found that 100 and 1000 p.p.m. of aldrin increased molds to a high peak at 5 days, after which a decline resulted.

In laboratory experiments with two soils having pH values near 8 and differing widely in fertility, Jones (70) observed that several chlorinated hydrocarbon insecticides were not toxic to ammonification below 1000 p.p.m. Aldrin, dieldrin, and 1,2,4,5,6,7,8,8a-octachloro-4,7-methano-3a,4,7,7a-tetrahydroindane (chlordane) were more toxic to nitrification than were BHC, DDT, endrin, or methoxychlor, inhibiting the process at 100 p.p.m. Sulfur oxidizing bacteria were less resistant than ammonifiers, and reacted to the various toxicants to about the same degree as did the nitrifiers. Toxicity of the compounds was generally less in the soil that was higher in organic matter.

On field-treated sandy loam and sandy clay loam soils, Martin *et al.* (94) found that neither aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, lindane, nor toxaphene (chlorinated camphene) at maximum rates normally used on field soils had any demonstrable effects on bacteria, fungi, or the functioning of the soil population in organic matter decomposition and ammonium oxidation.

Verona (127) could detect no influence exerted by 22 p.p.m. of BHC on the total numbers of bacteria in soil 10 days after treatment. Development of *Azotobacter*, nitrifiers, and cellulose decomposers on silica gel plates was increased.

Gray & Rogers (48) observed that the presence of BHC at approximately 125 p.p.m. in agar plates used for estimating bacteria and actinomycetes in soils prevented the development of more than 90 per cent of these organisms appearing as colonies on controls. Only Gram-negative short rods grew on the treated plates. In later tests with 50 species representing a wide range of heterotrophic bacteria, 13 grew on nutrient agar containing 250 p.p.m. BHC, of which 12 per cent was the *gamma* isomer (lindane). Thirty-five strains that did not grow in the presence of the BHC were not affected by the *gamma* isomer at 30 p.p.m. (47).

Gray (46) noted that while BHC up to 400 p.p.m. was toxic to nitrifiers and urea bacteria in solution cultures inoculated with soil, it was not inhibitory to nitrification of ammonium sulfate and urea in soil itself or in solutions to which plant organic matter was added.

Data obtained by Eno & Everett (35) one month after applying 12.5, 50, and 100 p.p.m. of ten chlorinated hydrocarbon insecticides including aldrin, BHC, and DDT, to a loamy fine sand soil showed that numbers of bacteria were not affected. Only dieldrin influenced the numbers of fungi, giving small increases. Carbon dioxide evolution was not significantly changed by any of the toxicants, but nitrate production was decreased by BHC. Wegorek (135) observed no significant changes in the number of bacteria, fungi, and *Radiata* in soil treated with chlordane at 220 p.p.m., or with BHC at 175 p.p.m. In laboratory and lysimeter studies with silt loam and sandy loam soils, Shaw & Robinson (119) observed no inhibition of nitrification of ammonium nitrogen by aldrin, heptachlor, and 2,4-D in amounts to 50 p.p.m., or by chlordane up to 150 p.p.m. active ingredients. Aldrin, chlordane, and lindane used at 45 and 450 p.p.m. in soil reduced the development of *Ophiobolus graminis*, *Pythium sp.*, and *Rhizoctonia crocorum* on beet, carrot, and wheat under greenhouse conditions [Grossmann & Steckhan (50)]. Toxicity to the fungi was greatest with chlordane, and least with aldrin. *Pythium* was less sensitive than was *Rhizoctonia*, while *Ophiobolus* was more sensitive. At normal insecticidal concentrations, chlordane considerably lowered the development of *Ophiobolus*, but the other compounds increased the attack by *Pythium*. The insecticides had much greater antifungal effect on agar plates, beginning at 1 to 10 p.p.m.

Braithwaite *et al.* (13) found that nodulation of clover on sod was greatly reduced by the application of DDT at 10 lb. per acre or of lindane at 1.5 lb. per acre in a fertilizer mixture. Aldrin, dieldrin, or chlordane similarly applied had only slight effects.

Wilde & Persidsky (137) observed that BHC, chlordane, allyl alcohol, and other biocides used on forest nursery soils, affected the development of fungi symbiotic on roots of *Pinus radiata*. The shape of the fungal mantle and other mycorrhizal characters were altered. As a result of external modifications of the short roots, radical changes in the rhizospheric exudates seemed probable.

It has been observed that many of the chlorinated hydrocarbon insecticides, with the exception of endrin, disappear from the soil at appreciable rates [Foster *et al.* (42)]. BHC is the least persistent, and many heterotrophic bacteria will grow on nutrient media containing 250 p.p.m. of this toxicant (48). However, microorganisms have not been directly implicated with the disappearance of any of these toxicants from the soil.

Because the anomalous insecticidal behavior of aldrin and dieldrin in Oregon field trials indicated some deterioration or transformation, studies were made to determine whether or not microorganisms were involved. Kiigemagi *et al.* (72), using the total chloride and the mosquito larvae bioassay methods, obtained a high initial recovery of aldrin, dieldrin, and heptachlor from field-treated soil but subsequently found a rapid decline. Bollen *et al.* (12) made microbial and chemical analyses on successive samples from

specially designed and prepared plots of Chehalis and Olympic silt loam soils treated with aldrin and dieldrin, at nominal rates of 5 p.p.m. The specific phenylazide method revealed that after 21 months nearly one-half of the 5.4 p.p.m. of aldrin originally applied was lost, although a significant amount was recovered as dieldrin. Dieldrin loss was less than that of aldrin from plots treated with the respective insecticides. Because traces of aldrin were found in dieldrin-treated sterilized soil, it appeared that non-microbial transformation occurred. Laboratory studies on the same soils with aldrin and dieldrin purified by recrystallization indicated that microbial activity was little involved in these changes.

An investigation by Lichtenstein & Schulz (81) on the transformation of aldrin to dieldrin, the epoxide, showed that while the change was appreciable in moist soil it was small in dry soil, or in soil poor in microorganisms. The epoxidation, apparently biological, was less rapid with heptachlor. Two major factors affecting persistence of aldrin, DDT, and lindane appear to be the amount of organic matter in a particular soil type and the climatic conditions of the area (80). Mader (86) demonstrated that the incorporation of biologically active "humus" in forest nursery soils could reduce the deleterious effects of various biocides used in nursery practice.

Organic phosphates.—Parathion, a highly toxic but short-lived insecticide, is rather rapidly decomposed by bacteria in soil and stimulates the increase of various physiological groups, including nitrifiers as well as nitrogen fixers [Naumann (103)]. Only excessive applications (250 p.p.m.) are inhibitory. Decomposition products of parathion apparently stimulate certain microorganisms in soil. This worker (102) observed peaks of increase in microbial numbers at 7, 12, and 22 days in chernozem treated with 10,000 p.p.m. of parathion, the increases being greater with moisture at one-half field capacity than at higher or lower values. In a clay loam soil under greenhouse conditions, applications to 50 p.p.m. had no effect on nitrifiers, cellulose-decomposers, or other groups of soil microbes determined by Kasting & Woodward (71).

Ahmed & Casida (2) found that certain bacteria, yeasts, and algae rapidly absorb O,O-diethyl (S-ethylmercapto-ethyl) phosphorodithioate (thimet) and then release it slowly from living and dead cells in cultures containing 1000 p.p.m. of the phosphates as emulsions. Phosphorodithioate sulfoxides were more stable than the sulfides in the presence of the organisms. *Chlorella pyrenoidosa* oxidized thimet to the phosphorodithioate sulfoxide and, more slowly, to phosphorothiolate sulfoxide. Both the algae and *Torulopis utilis* oxidized the sulfides to sulfoxides. *Pseudomonas fluorescens* and *Thiobacillus thiooxidans* hydrolyzed thimet but failed to oxidize it; *T. thiooxidans* could not utilize sulfur from the molecule.

Octamethyl pyrophosphoramid (pestox), also known as OMPA and schradan, a systemic insecticide, was found by Verona & Picci (128) to have stimulating rather than adverse effects on the microflora of soil when applied

at rates as high as 3000 p.p.m. The numbers of *Azotobacter* were considerably increased. Tests *in vitro* on a number of bacteria and fungi showed stimulating effects with 100 to 500 p.p.m. Apparently some of the organic phosphorous of the compound was mineralized in the soil by microorganisms.

Bacterial insecticides.—Bacterial insecticides have recently been reviewed by Heimpel & Angus (55). In contrast to disease caused by an entomogenous bacterium, a bacterial insecticide is a poisonous substance produced by a bacterium not necessarily growing in the insect. *Bacillus thuringiensis thuringiensis* produces a potent insecticidal toxin, crystals of which appear in the sporulating cells. Within the past few years certain firms have been growing these crystalliferous organisms with a good yield of toxin. The harvested bacteria, extended with bentonite or other materials, are then used as dusts or sprays, and are highly specific for certain noxious insects. An especially desirable attribute of the bacterial insecticide is that it leaves no residues harmful to warm-blooded animals or desirable insects. Any effects on soil microorganisms have not been reported.

Inorganic Pesticides

The total annual consumption of inorganic compounds used for pest control is tremendous. Arsenic compounds, borates, and chlorates are applied to soils at rates of 500 to 2000 lbs. per acre as non-selective weed killers or soil sterilants. Although these may be inhibitory, it is doubtful that they ever sterilize the soil with respect to the total microbial population.

Sodium chlorate applied for weed control to manure or compost heaps has been found to undergo rapid decomposition under anaerobic conditions, apparently being decomposed by denitrifiers [Jansson & Torstensson (61)]. Under aerobic conditions slow reduction and consequent toxic effects on the microflora occurred. When manure treated with chlorate was applied to soil, nitrification was delayed (62). On a clay soil at 800 to 2400 lbs. per acre it had no effect on numbers of bacteria, actinomycetes, or fungi as determined by plate counts [Hoover & Colmer (60)]. Sodium chlorate at 2000 p.p.m. inhibited respiration of *Azobacter* in Warburg studies made by Magee & Colmer (87); its effect was equivalent to that of 2,4,5-T, and greater than that of 2,4-D.

Elkan & Moore (33) applied partial sterilizing agents at various rates to soils in greenhouse and field trials, then determined the effects on microorganisms by making differential plate counts and measuring CO_2 evolution by the closed flask method. Bichloride of mercury and calcium hypochlorite at 25 and 50 p.p.m. reduced the total microbes, being particularly depressive to fungi. The hypochlorite gave an initial stimulatory effect on total counts and carbon dioxide production at three days in the greenhouse soil. For treated field soils at 90 days and 13 months, total counts and fungi were considerably lower, but each chemical generally had a minor effect on respiration.

Lees & Quastel (76), using the soil percolation technique, demonstrated

that chlorate at 10 p.p.m. selectively suppressed nitrification of nitrite to nitrate under these artificial conditions. In studies with Honeoye silt loam incubated in flasks, Otten *et al.* (107) found that polybor-chlorate at 40 and 80 p.p.m. decreased nitrification more than dalapon and several other herbicides used at their respective field rates. Although both steps of nitrification were retarded, even the higher rate did not halt the process completely.

Surface horizons of orchard soils where intensive spraying programs have been practiced for many years show accumulations of arsenic, copper, and lead in concentrations excessive with respect to amounts in the native soil. The arsenic often becomes toxic to cover crops, especially alfalfa. Greaves & Carter (49) showed that sodium arsenite, the form used for weed control, up to 200 p.p.m. arsenic equivalent, greatly increased the numbers of bacteria in the soil. It lowered ammonifying power except in manured soil, and was highly toxic to nitrifiers. On the other hand, arsenates, used in orchard sprays, may be stimulating and can accumulate in comparatively large quantities without injuring beneficial microflora. Manns & Russell (91) reported that copper sulfate stimulated *Azotobacter* and ammonifying bacteria, especially *Pseudomonas*, in a Maryland soil. However, Watson & Bollen (134) noted that only 11 to 30 p.p.m. of copper were definitely inhibiting to bacteria in lake bottom samples. Sulfamic acid and ammonium sulfamate are herbicidal, and amounts to 500 lb. per acre may be employed for temporary soil sterilization. Bollen (9) obtained data indicating that the acid is slowly oxidized in soil to nitrate and sulfate. At 3020 p.p.m. of ammonium sulfamate, equivalent to 1000 p.p.m. S, it initially increased mold counts and lowered bacterial counts while lowering the pH only slightly. As measured by CO_2 evolution, general microbial activity in soil plus sulfamic acid was greatly depressed.

Polysulfides and thiosulfate, used in fungicidal and insecticidal sprays, and elemental sulfur, which for centuries has been used as a fungicide, are readily oxidized in soil by *Thiobacillus*. The resulting sulfate can augment the available supply of this often deficient plant nutrient.

Practical Considerations

Any of the various chemicals used as pesticides are capable of inhibiting or destroying at least certain soil microorganisms under certain conditions. Investigations so far, however, indicate that when used at recommended field rates these toxicants will not significantly affect microbial activities that are important in soil fertility. Despite this fortunate circumstance, newly introduced compounds should be investigated insofar as feasible under field and laboratory conditions. The great number of different pesticides now available render impractical the testing of all for possible harmful effects on desirable soil microbes. Perhaps, when the physical and chemical properties of soil can be correlated with behavior of pesticides of known composition and molecular structure, deleterious influences on fertility can be predicted. Hueppe's principle should also be borne in mind: "Every sub-

stance which in definite concentration will kill protoplasm, will inhibit development in smaller amounts, and in still greater dilution act as a stimulant." This, and the production of adaptive enzymes, may account for many of the effects observed.

In our chemical warfare against a multitude of noxious organisms, it is necessary to avoid serious injury to the great variety of microbes whose functions are vital to the crop-producing power of the soil. Factors that can contribute to their survival and performance of essential activities are microbial adaptability, adequate soil management, and use of the minimum effective dosage of the pesticides.

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THE FAMILY BRUCELLACEAE IN VETERINARY RESEARCH[1,2]

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Let us begin by defining the field we intend to deal with. In this review the term "veterinary research" simply means investigative work that has contributed to the understanding of animal disease problems, in this case as related to the family Brucellaceae. Such work includes much basic research on animal pathogens, even when it cannot yet be correlated to any pathogenic activity or to such practical aspects as epidemiology, chemotherapy, and immunization. The term "Brucellaceae" is used in conformity with *Bergey's Manual of Determinative Bacteriology* although the writers have considerable doubt as to the merits of this innovation. We agree with Heidelberger's dictum: "If you try to draw too definite a picture of something you don't know much about, then you are pretty sure to be wrong" (67). We believe that the name "Brucellaceae" attempts to draw such a picture and conveys an entirely erroneous impression of this highly diverse group of organisms, for twenty years so aptly described as "Parvobacteriaceae" (122).[3]

This review concentrates on the more recent developments in research on *Brucella*, the pasteurellae affecting domestic animals, *Haemophilus* and *Haemophilus*-like forms, and the etiological agent of epididymitis in rams, whose suggested name (*Brucella ovis*) is not apt to gain acceptance among *Brucella* workers. The agent will very likely be assigned to some genus of the family Brucellaceae.

BRUCELLA

Because of the importance of *Brucella* in diseases of animals and man, we find for that genus a great variety and volume of research activity. The nature of brucellosis, including its interesting history, has been competently discussed by Spink (141). The present review is confined chiefly to recent

[1] The survey of the literature pertaining to this review was concluded in January, 1961.

[2] The following abbreviations will be used: BRT (Brucellosis ring test); DNA (deoxyribonucleic acid); DPN (diphosphopyridine nucleotide).

[3] While the new family designation is apparently intended to bring the nomenclature into line with rule 3 of the International Code of Nomenclature of Bacteria and Viruses, i.e., to derive the family name from the name of the type genus, we plead for the same special dispensation still enjoyed by the *Enterobacteriaceae*, a family much more easily represented by a type genus than the *Brucellaceae*.

work on characterization of the species within the genus *Brucella*, and on control and eradication programs.

The term "brucellosis" has been applied to the disease caused by *Brucella* spp. The chief susceptible animals, besides man, are goats, cattle, swine, and, where they are used as milk animals, sheep. The condition is usually characterized in these domestic livestock by epidemics of abortion, and in man by a variety of symptoms, chiefly intermittent fevers, night sweats, and muscular and arthritic pains. The chief reservoirs from which infection is readily transmitted are goats, cattle, swine, and sheep. There are no authentic reports of transmission from man to other susceptible hosts. Infection in horses is reported occasionally, usually in the form of a suppurative atlantal or supraspinous bursitis. Little is known regarding the epidemiology or pathogenesis of the condition in the horse; it is probably influenced by a primary injury, and is unimportant in the over-all picture of brucellosis.

There is ample evidence that hares are an important source of infection in some countries. From an infected hare in Germany, Witte (164) isolated a strain that he identified as *B. abortus*. Strains have also been isolated from hares by Nižnánsky *et al.* (116) in Slovakia, by Bendtsen *et al.* (10) in Denmark, and by Tudoriu *et al.* (157) in Rumania. Fritzsche (57), in a serological examination of 993 hares in Germany, found that 6.75 per cent of them reacted to the blood agglutination test. Stoenner & Lackman (150) isolated a strain of *Brucella* from a wood rat in the United States.

The volume of evidence clearly demonstrates the importance of wild rodents as foci of infection. This reservoir is undoubtedly a factor in the epidemiology of brucellosis in swine. Some interesting observations were made as to the identity of strains isolated from hares, particularly in Denmark. These are discussed herein under classification of species within the genus *Brucella*.

There is slight serological evidence that deer may be susceptible hosts. Ostertag & Mayer (118), studying sheep dogs as reservoirs of infection, found the organisms widely distributed through the body, but chiefly in the lymph nodes of the head, tonsils, and salivary glands. Serological tests were apparently unreliable in the dog. They considered that dogs were capable of perpetuating infection in herds of sheep.

The chief emphasis in this review is on the veterinary aspects of brucellosis, but reference should be made to recent work on human brucellosis. An unusual opportunity was available at the Johns Hopkins University School of Medicine to study a number of proven cases in which clinical and serological histories were available prior to exposure. Sixty cases, occurring between 1945 and 1957 in laboratory personnel engaged in work on *B. melitensis* and *B. suis*, were reported on in a series of three papers. Trever *et al.* (155) dealt with the laboratory-acquired acute infection, discussing it from the standpoint of age, sex, race, symptoms, physical findings, fever range, blood picture, hepatic function, serology, and therapy. Cluff *et al.* (45)

dealt with the medical aspects of convalescence in a study of 24 patients who had had acute brucellosis four to eight years previously. Sixteen of these subjects, in the category "chronic brucellosis," reported a variety of symptoms. The authors noted: "The resemblance of 'chronic brucellosis' to neurosis, therefore, suggested that psychologic factors might be of importance in the pathogenesis of this disorder." Imboden *et al.* (77) discussed the psychologic aspects of delayed convalescence, supporting the view " . . . that emotional disturbance is the essential component of 'chronic brucellosis.' "

Spink (140) reported on the significance of bacterial hypersensitivity in human brucellosis. In this he included studies on infection with strain 19 of *B. abortus*. Studies of this strain are of particular importance since, because of its low virulence for cattle, it is used extensively as live vaccine against bovine brucellosis. Four cases of human brucellosis caused by this strain were discussed by Spink.

The incidence of human brucellosis is extremely difficult to estimate. McCullough (96), from reported cases in the United States from 1927 to 1956 (84,754 cases), noted a steady increase from 1927 to 1947. Since then there has been a sharp decrease. The increase between 1927 and 1947 cannot be explained by any increase in domestic animals. As stated by this writer (96), the increase is presumed to reflect a growing awareness of the disease by physicians rather than an actual increase. He attributes the subsequent decrease to more widespread use of pasteurization and intensified eradication programs in domestic animals. The report includes incidence reported by states and districts between 1947 and 1956. All states are represented during that period. The reported number of cases was 6321 in 1947, and 1080 in 1956.

These data are based on cases reported by physicians. The majority are probably supported by laboratory findings; others are probably based on clinical evidence only. It is to be expected that many cases of brucellosis are not diagnosed as such, chiefly because of the unreliability of the routine diagnostic procedures employed. The source of human brucellosis is infected animals or their raw products. The disease is an occupational hazard encountered chiefly in meat-packing houses and by veterinarians and other livestock handlers. Hendricks *et al.* (69) reported an outbreak of human brucellosis among employees of a packing house—128 proven cases over a nine-month period. Only pork was being processed at that plant. *B. suis* was isolated from seven patients, and *B. melitensis* from twelve. The identity of the latter is discussed later herein. Drankin (48) made an epidemiological analysis of the seasonal cyclic occurrence of brucellosis in Orenburg Oblast, in the U.S.S.R. The analysis was conducted on three groups: (*a*) animal raisers, (*b*) rural population not connected with animal raising, and (*c*) urban dwellers. There was little difference between the first two groups, but in the third the incidence, as expected, was significantly lower. In groups (*a*) and (*b*) the incidence reached peaks in March and May, respectively, coinciding

with the handling of animals in routine management practices. In group (*c*) the peak was in July. *B. melitensis* was isolated exclusively, and in 90 to 95 per cent of all cases the sources of infection were sheep and goats.

Of considerable interest is the intensive bovine brucellosis eradication program conducted by the United States Department of Agriculture in co-operation with the states. The uniform rules and methods under which the program operates are set forth in the report of the brucellosis committee of the United States Livestock Sanitary Association (25). Basically, the program is one of vaccinating calves between the ages of four and eight months with living strain 19 of *B. abortus*, together with a blood test of adult cattle and the slaughter of reactors to the blood agglutination test.

A progress report by Mingle (111) showed that, as of November 30, 1959, out of a total of 3152 counties in the United States, Puerto Rico, and the Virgin Islands, 57 per cent had acquired the status "Modified Certified Brucellosis-free." Nineteen entire states are now in this category. This status is attained when the incidence of infection is reduced to not more than 1 per cent of the cattle in not more than 5 per cent of the herds in the area. When no infection exists, the state is declared "Certified Brucellosis-free." One state (New Hampshire) has been so declared. According to Mingle (111), annual losses from bovine brucellosis have been reduced from 100 million dollars in 1947 to less than 30 million dollars.

Diagnosis of bovine brucellosis has been the subject of considerable research. The brucellosis ring test (BRT), using a composite herd milk sample, is widely employed to detect infected herds. The test, which depends on the presence of specific agglutinins in the milk, is extremely sensitive. It is used in the United States on a composite whole milk sample taken from as many as 150 cows. A hematoxylin-stained antigen is added, and the sample is incubated for 1 hr. If agglutinins are present the antigen is agglutinated and rises with the cream layer to color it deeply. The remainder of the sample reverts to normal color. The sensitivity of the test is in direct proportion to the number of cows represented in the sample and to the agglutination whey titers of the infected cows. On occasion, the reviewers have encountered positive BRT reactions with only one infected animal among 200 milking cows as determined by blood or whey test of individuals in the herd. The method was first described in Germany by Fleischhauer (56), and in the United States by Roepke *et al.* (128).

The blood agglutination test is then used to detect the infected animal in the BRT-suspicious herd. The interpretation of blood titers is based on whether or not the animal had been vaccinated as a calf. A higher titer is tolerated in a vaccinated animal than in an unvaccinated one. High blood titers do not persist as a rule in calves vaccinated under eight months old, but except in registered animals, a calf's age is often a matter of conjecture. Some titers will persist in the suspicious range to create a problem in interpretation and raise the question as to the specificity of the reaction. At-

tempts have been made to differentiate the specific from the nonspecific reaction. For this purpose an acidified plate antigen was suggested by Stiles *et al.* (148). Smith *et al.* (138), in 1923, stated that there was an active participation by the udder in agglutinin production when invaded by living, or flooded by dead, bacteria. Cameron & Kendrick (36) therefore advocated a whey test to differentiate blood reactions resulting from vaccination from those caused by virulent strains. This is based on the observation that the vaccine strain does not, as a rule, localize or persist in the mammary gland, and that there is no local production of antibody. Cameron (33) advocated greater use of the whey test in the bovine brucellosis eradication program, chiefly because of its simplicity and practicality in areas where the obtaining of frequent blood samples would be costly. Stiles *et al.* (148) and Janney & Berman (78) concluded that the test had serious limitations, however, because they isolated *B. abortus* from cows that reacted in the blood but not in the whey test. Their investigations, however, were in areas where blood test and slaughter had been under way for a number of years, and infection in the herds could well have been of too recent origin to have caused the development of antibodies in milk. In such instances, all serological tests have limitations. Further, Janney & Berman whey-tested only the blood reactors in BRT-suspicious herds. Their conclusions would have been sounder had they also examined the blood-negative animals. These workers reported 8000 herds, suspicious on the basis of the BRT yet containing no reactors to the blood test. The herds in question should have been whey tested too because the BRT reaction is caused by agglutinins in the milk rather than in the blood. One is not justified in calling the herd BRT "falsely suspicious" on the basis of a negative blood test of individual animals. Cameron (33) reported a few animals that were negative to blood and positive to whey tests when their milk contained virulent *B. abortus.* He also reported a highly significant number that were only suspicious to the blood test but positive to the whey test, and were proved to be infected on culture of milk. Milunović (110), on the basis of a parallel study of rapid milk agglutination tests with blood serum tests, recommended that the former be undertaken more frequently in chronically infected herds, and that positive reactions be compared with the results of blood serum tests for improved efficiency in eradicating bovine brucellosis. Bürki (30) favors the complement-fixation test, claiming that it is more sensitive than serum agglutination and compares favorably with the whey test. He does not consider cream culture sufficiently sensitive as a diagnostic test. In Switzerland, whey reactors are condemned even if negative on cream culture. Sackman (129), reporting 108 virulent strains isolated from animals vaccinated with strain 19 of *B. abortus,* attributed the discrepancy to a breakdown of immunity or early infection. We do not think this represents a breakdown in immunity; vaccination with strain 19 produces only a local immunity (in the genital tract) to prevent abortion. It does not prevent infection if the animal is

exposed. In such instances, localization is usually in the mammary gland. A comparable situation is found in natural epidemics in that an animal, as a rule, does not abort a second time from brucellosis.

It is unfortunate that a comparable eradication program in swine has not yet been initiated on a national basis. Swine brucellosis causes heavy losses through abortions when infection first invades breeding herds. It is also an important factor in public health, as evidenced by recent findings of Hendricks *et al.* (69). Brucellosis in swine was recently described in detail by Manthei (103). The condition was first reported in the United States by Traum (154), in 1914. Since then it has been reported from a number of countries where swine are raised. A notable exception is Great Britain, where apparently there is no brucellosis in swine. Several epidemics in Denmark have been reported by Bendtsen (10). The interesting feature there is that the hare is an extremely important source of infection. It is also of interest that the strain of *B. suis* isolated in Denmark varies in biochemical behavior from the *B. suis* usually associated with brucellosis of swine in the United States. *B. suis* from Denmark is called *B. suis* type II by Huddleson (70). This feature is discussed below under the classification of the species.

Serious economic losses result when a susceptible herd is exposed. Cameron (32), in comparing production in infected and uninfected groups of breeding swine, observed that, based on the number of sows bred, two pigs per litter were weaned from the infected group, as compared with eight from the uninfected. Responsible for much of the swine brucellosis in the United States is latent infection in purebred herds which are sold as young breeding stock to commercial pork producers. Hutchins & Andrews (75) consider the boar as playing an important role in transmitting swine brucellosis. Equally important in epidemiology is the aborting young female.

Eradication of brucellosis from herds of swine depends essentially upon blood tests and the slaughter of infected animals. The brucellosis committee of the United States Livestock Sanitary Association (24) has recommended a choice of several plans, depending on the type of herd operation. The diagnosis of brucellosis in swine presents difficulties. Cameron & Carlson (34) concluded that a negative blood agglutination test did not mean freedom from infection in the individual pig in an infected herd. Cameron (31) therefore recommended that the test be interpreted on a herd basis and that exposed animals, even when negative to the blood test, be considered infected. The blood reactors and exposed animals would be considered infected and held in isolation until disposed of. Cameron *et al.* (35) produced evidence suggesting a genetic resistance to brucellosis in certain strains of swine. It is unfortunate that these investigations were not continued. Hutt (76), in his book on genetic resistance to disease, discusses this phase of swine brucellosis.

Classification of the species.—The basis for classifying these organisms is extremely confusing. Three species are generally recognized at this time—*B.*

abortus, *B. suis*, and *B. melitensis*, with the respective normal hosts being cattle, swine, and goats and sheep. From the standpoint of virulence, the host-parasite relationship is fairly specific, although *B. abortus* in swine was reported by McCullough *et al.* (97), *B. suis* in cow's milk by Jordan & Borts (82), *B. melitensis* in cattle by Stableforth (143), and *B. melitensis* in swine by Borts *et al.* (19), Jordan *et al.* (83), and Kabler *et al.* (84). Thus, it appears that any of the hosts may serve as a reservoir for any of the species without evidences of clinical brucellosis. Man is susceptible to all three species. Classification cannot, therefore, be based upon the finding of an organism within a particular host. Differentiation of species as recommended by Huddleson (71) depends on the bacteriostatic action of thionin, which inhibits the growth of *B. abortus*, and basic fuchsin, which inhibits the growth of *B. suis*. *B. melitensis* is not inhibited by either dye in the concentrations employed. *B. abortus*, on primary isolation, usually requires the addition of CO_2 to the environment for growth, though this characteristic is not invariable. Bouvier (20) stated that 12.4 per cent of *B. abortus* strains tested did not require CO_2 on primary isolation. The production of H_2S is another criterion employed in differentiating the species: *B. melitensis* fails to produce it, *B. abortus* produces it in moderate amounts, and *B. suis* is a heavy producer. Again, these reactions are variable.

These are considered the most reliable criteria, and most cultures are readily classified by them. Strains are encountered frequently, however, that cannot be classified by conventional methods. Serological typing is sometimes attempted, but this has limitations and is frequently in opposition to biochemical typing. The methods involve the use of monospecific antiserum prepared by careful absorption wlth heterologous cells. Wilson & Miles (162) showed that there were two main groups antigenically: (*a*) *B. melitensis* and (*b*) *B. abortus* and *B. suis*. They emphasized that the use of an absolutely smooth strain was imperative or the results would be confusing. It was concluded that all species contained the same antigens A and M, but that there was a marked quantitative difference among them. Von Sprockhoff & Strauch (160) studied a number of strains with respect to their A and M antigens and concluded that, if type-specific sera were used, *B. melitensis* could be distinguished from *B. abortus* provided the strains were freshly isolated. They also pointed out that old strains of *B. melitensis* are likely to change their antigenic structure. It thus appears that classification on the basis of serology should be approached with caution, and the consensus is that classification based on serology alone is unsatisfasory. Renoux (124) encountered organisms with biochemical characteristics of *B. abortus* and serological behavior indicative of *B. melitensis;* he suggested a new species: *B. intermedia*. An excellent account of the present confusion can be found in a discussion by Stableforth (142).

The species have been further divided into types by Huddleson (70) who discusses them as to origin. Thus, we now have *B. abortus*, types I, II, and

III; *B. suis,* types I, II, and III; and *B. melitensis,* type I. The members of type I are considered typical of the species and are readily differentiated by their dye tolerance; the other types show variations in characteristics which may be related to geographical distribution. *B. abortus,* type II was first isolated from cattle in France. It appears to come chiefly from Asia but has been reported from the United States by Huddleson & White (72) and Goode *et al.* (59). *B. abortus,* type III has a wide distribution, but there is no record of its isolation in the United States. *B. suis,* type II is usually referred to as "Danish" *B. suis,* and *B. suis,* type III is sometimes called "American" type *B. melitensis. B. suis,* type III has been isolated from swine in the United States, and it may possibly be the type Hendricks *et al.* (69) recently found in Iowa in cases of human brucellosis following exposure to infected swine. From Huddleson's (70) classification of the species and types, it can be observed that *B. suis,* type III cannot be differentiated from *B. melitensis* by dye tolerance, H_2S production, or CO_2 requirement. The same can be said of *B. abortus,* type III, in which a difference in CO_2 requirement is not constant. Pickett & Nelson (120) suggest distinguishing the species and characterizing "species-intermediate strains" on the basis of fermentation of inositol, maltose, mannose, rhamnose, and trehalose. Renoux (125) expressed doubt as to the value of these criteria.

Cameron & Meyer (37) attempted to change the characteristics of *B. abortus* strain 19 by serial passage through swine. A residency of 406 days in 26 pigs produced no observable change in virulence or dye tolerance. Serological methods were not used in this work. It should be pointed out, however, that strain 19 *B. abortus* is extremely stable. Karsten (85) stated that differentiation should be based upon pathogenicity for a particular host, and claimed that reliance on current biochemical and serological methods leads to confusion. Even so, Borger (18) transmitted *B. melitensis* from infected cows to goats.

Renoux (125) recommended establishing a single species subdivided into varieties or types. He based this view on the lack of qualitative differences among the three species, the wide host range, the lack of agreement between serological and biochemical tests, the probability that simple mutational change may occur, and the difficulty in classifying many strains under the three species. Van Drimmelen (158) considered the taxonomic situation unclear, and he also suggested that there is only one species in the genus. He would have *B. melitensis,* with varieties *thomseni* (Renoux, 1952), *lisbonei* (Renoux, 1952), *intermedia* (Renoux, 1952), and *karakul* (Van Drimmelen). The use of varieties rather than species would still create a problem in taxonomy. This is of more than academic interest in that identification is fundamental to establishing the epidemiology of brucellosis. Classification of the genus into three species by Huddleson (71) in 1929 was confirmed serologically by Wilson & Miles (162), and was upheld as biologically sound by Meyer & Cameron (108) on the basis of oxidative metabolic patterns.

Buddle & Boyes (29) isolated an organism causing epididymitis in rams,

and suggested the name *Brucella ovis* for it. The name has not yet been universally accepted. Meyer & Cameron (106) suggested, on the basis of serology and oxidative metabolism, that it not be included in the genus *Brucella* (see below). Stoenner & Lackman (150) isolated from the wood rat an organism that they designated *Brucella neotomae.* It is not dependent on CO_2, and produces H_2S as does *B. suis*, but it grows on basic fuchsin and thionin. The oxidative metabolic pattern as examined by Cameron & Meyer (38) does not conform to that of the other three species. Beal *et al.* (9) exposed young pigs to this organism intravenously, and were able to establish infection in all as determined by blood agglutination and persistent bacteremia. No other evidence of brucellosis was apparent in these animals.

Stinebring & Braun (149), using brucellaphage *abortus*, type III obtained from the U.S.S.R., showed that, when tested against a number of strains of all species, only smooth and intermediate strains of *B. abortus* proved sensitive to it. Jones (81) and Brinley-Morgan *et al.* (23) have also reported work with phage. This additional tool should be of considerable value in identifying *B. abortus.*

Meyer (105) critically examined the standard methods for differentiating the species, including H_2S production, growth on basic fuchsin and thionin, phage typing, and oxidative metabolic activity. The technique employed in the last method was that used by Meyer & Cameron (108). More than 200 strains of world-wide distribution were examined. Many of these cultures were designated atypical because of variations in dye tolerance, H_2S production, CO_2 requirement, or serology. Fifteen were *B. intermedia.* Included were 25 each of typical *B. abortus*, *B. suis*, and *B. melitensis.* Of particular interest in this extensive investigation were the data on oxidative metabolism. Eleven amino acid and nine carbohydrate substrates were used. The metabolic pattern was clearly defined for all species. By using asparagine, citrulline, ornithine, lysine, ribose, galactose, arabinose, and xylose, all cultures could be accommodated within the three existing species. The metabolic pattern was not influenced by dissociation, and it appeared to be independent of any change in dye tolerance. For example, *B. melitensis*, *B. abortus*, type III, and *B. suis*, type III, all tolerate basic fuchsin and thionin, yet they are clearly differentiated by the metabolic pattern. All strains defined as *B. abortus* by metabolic pattern were confirmed by phage sensitivity, even though they seemed to be *B. melitensis* in terms of dye tolerance and H_2S production. None in the other two species were sensitive to the *B. abortus* phage.

It thus appears that designating the species as *B. abortus*, *B. suis*, and *B. melitensis* is well justified. After all, the great majority of the cultures can be classified by standard methods and the variants are relatively few in numbers. Though manometry is tedious, the work of Meyer (105) indicates that it is the method of choice for positive identification of variant strains in the genus *Brucella.*

It would be inappropriate to omit mention, at least, of current research on

basic immunology. The subject of cellular immunity is well discussed by Elberg (50). Heilman *et al.* (68) investigated the specific cytotoxicity of the nucleoprotein fraction from *Brucella* species. Toxicity was determined by inhibition of migration and growth of leukocytes and fibroblasts, and by the morphologic appearance of stained sections of cultured cells following different periods of incubation in the presence of nucleoprotein. Elberg *et al.* (53) demonstrated a non-specific element in the resistance of monocytes from immunized rabbits. Vaccination with Bacillus Calmet-Guerin or brucellosis antigen induced protection against both *M. tuberculosis* and *B. melitensis.*

PASTEURELLA

The genus *Pasteurella* has occupied an ambiguous position in the field of veterinary microbiology. Originally credited with causing septicemic infection or acute pulmonary disease in wild ruminants and domestic mammals of economic importance, it was gradually demoted to the role of a secondary invader with a questionable part in the pathogenic process. The reasons for this lowered status were the inability of most strains to produce anything resembling the clinical disease, and the occurrence of typical strains on mucous membranes of normal animals. With the coming of the virus age, a diligent search has been instituted for viral agents to account for the diseases formerly thought to be caused by the *Pasteurella* species. This search has not been successful; or, rather, it has been too successful since, in various parts of the country, numerous viruses (102) ranging from psittacoid (119) to hemadsorbing (123) have been found to be associated with the pneumonic infection, particularly of ruminants, from which *Pasteurella* was regularly isolated. Some of the virus strains, reintroduced into the original host species, caused mild respiratory disturbances hardly comparable to those seen in the field (64). At this writing, a single specific viral cause of the respiratory and systemic diseases, with which *Pasteurella* is associated, remains as elusive as ever.

In the meantime, certain information about *Pasteurella multocida* has gone far in accounting for ambiguities observed in earlier investigations, and has secured a place for members of this species as primary pathogens. If one were to choose a point at which a beginning was made, it would have to be in the establishment by Roberts of four immunological types of this bacterium (127), since enlarged to five (73). Using passive immunization of mice to define types, Roberts showed that these correlated to some degree with predilection for species, with geographic distribution, and with biochemical behavior as shown largely by fermentative activity in xylose, arabinose, and dulcitol. Roberts' work was confirmed in a series of extensive studies by Carter and various collaborators. Substituting first precipitation tests and the Neufeld reaction (41) and then the indirect hemagglutination test (39) as typing procedures, Carter arrived at the same four types as had Roberts (although substituting the terms B, A, C, and D, respectively, for Roberts' I, II, III, and IV).

In applying the typing to strains from all over the world, Carter (40) drew further attention to a fact already suggested, though not emphasized, by Roberts' data: that the immunological type largely determined the clinical manifestation of pasteurella infection. This was particularly true of type I (Carter's B), the incidence of which is confined to the Old World, particularly southeast Asia. This type is responsible for the classical hemorrhagic septicemia found particularly in India, Ceylon, and Indonesia, where it causes great losses in bovines and has therefore been studied in some detail. The cells of this type are surrounded by a layer whose general chemical compositon and complexity are similar to those of the surface structure of *Pasteurella pestis* (4). Twelve capsular antigenic systems have been suggested by gel precipitation tests in Ouchterlony plates (6). Polysaccharides, proteins, and lipoproteins are concerned in the capsular structure (5). Capsular material is actively immunogenic, but the immunogenicity is abolished on deproteinization, and immune serum absorbed wlth isoelectric fractions loses its power to protect mice against type I infection. Polysaccharides also appear to have protection-inducing properties. When purified they are haptens that are capable of absorbing some of the mouse-protective antibody from immune sera. The polysaccharide fraction is abundant in the capsule of freshly isolated strains, which are inagglutinable in all but the lowest dilution of potent sera. The gradual disappearance of this inagglutinability is paralleled by a decrease in polysaccharides from the capsule. Lipopolysaccharides have some immunogenic activity. They are toxic and represent the antigenic fraction that modifies erythrocytes for indirect hemagglutination (5). Acid hydrolysates of these contained aldoheptoses, glucosamine, and occasionally glucose. This composition is reminiscent of lipopolysaccharides of rough *Pasteurella pestis* (95).

Bain (5) offers the opinion that none of the substances obtained by supposed extraction of the capsule needs to be exclusively capsular, since a clean, differential extraction is unlikely. Carter & Annau (43) showed that capsular preparations obtained by saline extraction under very moderate heat showed marked variations in nitrogen content, reducing activity, and phosphorus content, according to the colonial form from which they had been isolated. They pointed out that the mucoid strains differ from strains of other colonial forms in containing larger amounts of capsular hyaluronic acid, which is nonantigenic and nonimmunogenic. Carter interpreted dissociation to the mucoid form as an adaptive phenomenon that enables the bacteria to circumvent the immune mechanisms of the host. He found mucoid forms to be typically associated with chronic infections. Of the other colonial varieties, the smooth form is generally considered to be the most consistently pathogenic.

Differences in degree of virulence are correlated with a property described originally as fluorescence and more recently as iridescence. Elberg & Ho (52) reported three types of fluorescent colonies—green or golden, red, and blue—with decreasing virulence, in that order. Non-fluorescent colonies are of

questionable pathogenicity and they are also unsatisfactory for immunizing preparations. Although typability is maintained throughout the series and there are no demonstrable changes in cellular morphology, including encapsulation, there are antigenic differences among the several smooth colonial types. These differences, however, are believed to be quantitative rather than qualitative. The particular antigenic constitution of the fluorescent types is considered to be of importance in providing good immunizing preparations against hemorrhagic septicemia in Asia, since only bacterins derived from cultures showing this property have been of consistent value in preventing the disease. This is in line with the observation that fluorescent cultures are invaribly associated with this acute form of pasteurellosis (3).

Much less clear-cut is the situation with other forms of pasteurellosis. In the Western Hemisphere, particularly in North America, hemorrhagic septicemia is rare. We have seen it in calves, though associated not with *Pasteurella multocida* but with *P. hemolytica* (14). European reports do mention *Pasteurella multocida* as the cause of hemorrhagic septicemia in calves (94, 117). The serotype found in the Asian form is absent in America and at least Northern Europe (5); the serotypes in this country are less constant and clear-cut in their pathogenic properties. Types II and IV (Carter's A and D) are commonly found in pneumonias of cattle, popularly termed "shipping fever." They also occur in pneumonia of pigs as well as in diseases of birds, particularly in fowl cholera. Type C has been found predominantly in the domestic carnivores. Except in fowl cholera in which *P. multocida* plays a primary part but in which another species of *Pasteurella* may be involved in chronic cases (63), the role of *Pasteurella* in livestock diseases remains unsettled in the eyes of many students of the problem. Numerous investigations in recent years have attempted to reproduce the field condition in cattle (119) or sheep (64), using *Pasteurella* alone or in combination with viruses, pleuropneumonia-like organisms, and hormonally and physically induced stress. The results have varied from nil to subclinical to mild clinical infections, never the regularly severe and often fatal pneumonia that is characteristic of field outbreaks. Using the other agents without *Pasteurella* has given similarly disappointing results, and many students of the field, concluding that *Pasteurella* has no causal role in shipping fever, have assigned it the part of an adventitious secondary invader (21, 161). Their case is based not only on the failure of *Pasteurella* to produce the disease but on the frequent presence of *Pasteurella* on the respiratory mucous membranes of healthy animals. In our opinion, neither finding is evidence of a compelling nature. Numerous pathogens, human and animal, have their normal habitat on the mucous membranes of healthy individuals, as shown in the cases of pneumococci, meningococci, pathogenic staphylococci, hemophilic bacteria, and diphtheria bacilli. The question of experimental induction of disease in the natural host has, for obvious reasons, not been as thoroughly belabored in connection with human as with animal disease. That the organisms im-

plicated were consistently present in cases of the disease, that they elicited in experimental animals a disease response (though not always identical with that seen in human cases), and that their specific suppression regularly meant control of the disease, have long been accepted as satisfying Koch's criteria. It is generally recognized that predisposing factors are needed to establish pneumococcal and other bacterial pneumonias in man without disputing the causal role of the pneumococcus in bringing about the characteristic lesions, clinical manifestations, and possible complications. A close parallel is provided by *Pasteurella* and its part in the etiology of shipping fever. Pathologically, shipping fever is a typical bacterial infection, with its characteristic cellular inflammatory response and the severe extravasation of blood constituents resulting in hemorrhages, edema, ascites, and fibrin deposition. The often dramatic response to antibiotics and their value as prophylactic agents further demonstrates the important role of *Pasteurella* in the pathogenesis of the disease. There will probably be little argument against the proposition that the predisposing or primary factors in shipping fever appear to be highly variable and not necessarily even infectious. They might be characterized as non-specific. The bacterial factors are restricted for the most part to one genus whose pathogenic effects in experimental animals are, at the tissue level, qualitatively similar to the changes seen in shipping fever. The control of these organisms, according to all available evidence, means control of clinical shipping fever. To deny that they are agents of the disease appears to be almost a legalistic maneuver, outside the realm of biology.

PASTEURELLA HEMOLYTICA

If the place of *P. multocida* in veterinary microbiology has been called ambiguous, the term is even more applicable to *Pasteurella hemolytica*. The species is a relative newcomer, having been proposed less than thirty years ago (115), although the organism bearing this name was described in detail by Jones in 1921 (80). Its pathogenicity for experimental laboratory animals is lower than that of *Pasteurella multocida*. This fact, together with the difficulties of satisfying Koch's postulates with this organism, and the absence of its association with a serious epidemic disease like Asian hemorrhagic septicemia, caused this bacterium to be assigned a doubtful role in the production of animal disease. In recent years, attention has been called, particularly by Carter and co-workers (44), to the increased frequency with which *Pasteurella hemolytica* was found associated with shipping fever. Interest was aroused during the last ten years when, at short intervals, epidemics of septicemia in sheep were reported from such widely separated areas of the world as Norway (165), Scotland (146), Slovakia (151), and California (12). In the severest forms these outbreaks have many pathological and ecological features in common with the hemorrhagic septicemia of Asiatic cattle and buffalo, though the proportion of animals involved is usually much lower. In addition, septicemia of newborn animals has been

described in lambs (65) and pigs (49). In nearly all cases the organism isolated was *Pasteurella hemolytica*. The disease could be reproduced with reasonable regularity when very large doses of bacterial cells were inoculated parenterally. Smith (134) recently reported success with small inocula in the case of very young lambs. The etiological role of *Pasteurella hemolytica* in this septicemic disease is beyond reasonable doubt. Still being debated is its role in respiratory conditions, in both cattle and sheep; any valid pronouncement must very likely await a more thorough study of the organism itself.

Variations in cultural and in physiological and serological characteristics have been described. A colonial variation, designated S-R, recently reported (15), is accompanied by a loss of a surface antigen and reduction in virulence for mice. The biochemical activities of parent and variant strains were identical, as were the somatic antigens. In view of this later fact the designation M-S may be more appropriate.

Another type of variation, apparently quite different from the preceding one, was observed by Smith (136), who reported two types, A and T, varying from each other in fermentative activity, colonial morphology, penicillin sensitivity *in vitro*, growth and death rates, cellular-suspension stability in water, and the clinical conditions in which they were commonly found.

A serological study by Carter (42) of over 60 strains from cattle and sheep suggested that the species was antigenically uniform, but an investigation by Biberstein *et al.* (11) revealed 11 serotypes among a collection of 98 strains that were predominantly ovine in origin. The types, determined by indirect hemagglutination, appeared to be correlated to some degree with the nature of the disease condition, the host species from which they were recovered, and with certain biochemical activities of the strains. If these findings are confirmed, the pattern would closely parallel that already established for *Pasteurella multocida*.

No direct data are available on pathogenic mechanisms, but much of the evidence indicates that a toxic component associated with the bacterial cell is responsible for the pathologic changes and death in the septicemic disease. The numbers of microbes involved in such an infection are extremely large. Bacterial colonies are prominent in sections of lung and liver tissue in particular, where they are frequently found associated with blood or lymph vessels, and may give rise to secondary thrombi (12). In some groups of lymph nodes they fill the subcapsular space as solid masses. Stained impression films from the cut surface of such nodes resemble smears from fully grown broth cultures. In a recent investigation, Smith (135) determined the numbers of organisms present at death in various organs. Lung tissue contained 47 million to 14 billion living bacteria per gram of tissue; liver, 2 to 900 million; and spleen, 2 million to 22 billion. Smith reproduced the clinical and gross pathological condition by injecting heat-killed organisms. The numbers required to produce death were 10 to 50 times those needed with living cells. This narrow margin is strongly indicative of the toxic nature of the disease.

Earlier, Biberstein & Kennedy, though suspecting a toxic component in the infection in mice, failed to demonstrate toxicity in formalinized and heat-killed broth cultures. The dose they gave was only about 6 times the LD_{50} for living cells (12).

The situation is somewhat confused with regard to a mastitis of ewes from which a small Gram-negative coccobacillus is regularly isolated. The first mention of this bacterium is in a report in 1907 by Dammann & Freese (46), whose names have become attached to the organism in the German literature. In 1932, Miessner & Schoop (109) suggested the binomial *Bacterium mastitidis* as a substitute. More recently, Schröter (130) proposed renaming it *Haemophilus ovinus* because blood favors its growth. In 1932, Marsh (104) described the disease and its causative agent, proposing for the latter the name *Pasteurella mastitidis*. In the same year, Newsom & Cross (115) published their finding on *Pasteurella hemolytica*, but without regard to udder infection in sheep. Evidence appearing in recent years strongly indicates the close relationship, and perhaps identity, of the organisms of ovine mastitis with *Pasteurella hemolytica*. In Carter's serological study on *Pasteurella hemolytica*, referred to above (42), a number of strains of *Pasteurella mastitidis* were included and found to be serologically identical with the other *Pasteurella hemolytica* cultures used in this investigation. After a thorough study of 32 strains from mastitis and 5 from pneumonia as well as type strains of *P. multocida* and *P. hemolytica*, Todorov (153) concluded that *P. hemolytica* and *P. mastitidis* were identical. Reports from Norway (92), and Bulgaria (153) revealed the presence of *Pasteurella hemolytica* in ewe mastitis, whereas none of the other reports, including those of Dammann & Freese, Marsh, Miessner & Schoop, and a recent one by Simmons & Ryley (133) described any characteristic that would be a basis for separating the two species. An article by Smith & Hurnden (137) describes an outbreak in a particular flock. It is not clear from the bacteriological data how many isolates the findings are based on, and no mention is made of the most reliable criterion, i.e., indole production. Even so, the description clearly points to *Pasteurella multocida*, which was also the researchers' conclusion. This is almost certainly not the Dammann-Freese bacillus, Miessner & Schoop's *Bacterium mastitidis*, or Marsh's *Pasteurella mastitidis*, although Hudson (74) suggested such identity in a recent review of *Pasteurella mastitidis* in sheep.

In summary it may be said that probably many organisms, including *Pasteurella multocida*, are capable of causing mastitis in sheep, but that the *Pasteurella*-like organism most frequently described has most of the characteristics of *Pasteurella hemolytica* and is probably identical with it (63, 153). Only direct comparisons of cultures from various sources can give a definitive answer to this problem. Should the suspected identity be confirmed—as we expect it will—the fact that the species name *mastitidis* antedated *hemolytica* by several months will invalidate the latter designation and bring about more confusion in the nomenclature of this group.

Epididymitis in Rams

The organism responsible for epididymitis in rams has not been classified to the satisfaction of all. The designation enjoying widest currency, *Brucella ovis*, and the general resemblance of the organism to the members of the family Brucellaceae, make it an appropriate subject for discussion at this point.

Clinical epididymitis in rams was described in Australia in the early 1940's (60). The infectious nature of the disease was recognized at that time, but no progess in recovery or identification of a causative agent was made for another decade. In 1952 McFarlane *et al.* (98) reported on an infectious type of abortion among ewes in New Zealand and described coccobacilli that stained like elementary-body viruses and could be cultivated in the yolk sac of the chick embryo. The proposition that these bodies were, in fact, psittacoid virus particles was all the more plausible since Stamp and his group (145) had just reported the recovery of such an agent from aborting ewes in Scotland. In the same year, Filmer (54) reported findings tending to identify the causative organism of ewe abortion with that of ram epididymitis, and establishing its bacterial nature. The first detailed studies of the organism were carried out by Simmons & Hall (132), in Australia, and by Buddle & Boyes (29), in New Zealand. These investigations accounted for the elusive nature of the bacterium by showing the need for rich media, preferably containing blood or serum, and an atmosphere containing increased amounts of carbon dioxide for primary isolation and early passages. Isolation of the organism has since been reported from the United States (99), Czechoslovakia (58), Rumania (156), and the Union of South Africa (159). Bacteriological data in a German report (91) are so sketchy that it is impossible to decide whether a related organism was involved. The clinical description rather suggests that it was not.

Buddle & Boyes suggested that this organism was a non-smooth "stabilized mutant of *Brucella melitensis*" for which they proposed the name *Brucella ovis*. They regarded it as a brucella "on the basis of cell morphology, colonial morphology, cultural characteristics, biochemical properties, sensitivity to antibiotics and sulfonamides, pathogenicity for laboratory animals and the association with genital disease in the natural host." The basis of the species assignment was the behavior of the organism on dye media employed for the differentiation of *Brucella* species, and on the diethyldithiocarbamate agar of Renoux. Objections to the classification mentioned by the proponents themselves were: in contrast to *Brucella*, the sheep organism did not retain the primary stain in Koster's procedure; it was not agglutinated in *Brucella* antiserum; nor did it reduce nitrates or show any degree of urease activity. To these may be added the fact that the similarities quoted refer in part to atypical properties of *Brucella melitensis*. For example, typical *Brucella melitensis* tolerates methyl violet in a concentration of 1:100,000 and basic fuchsin in one of 1:25,000, whereas the ovine organism does not. Buddle &

Boyes pointed to strains of *Brucella melitensis* studied by Wilson (163) and described as "unduly susceptible to methyl violet." Wilson designated them *Brucella melitensis* on serological grounds only. Another characteristic inconsistent with the proposed classification is the carbon dioxide requirement exhibited by the epididymitis bacterium. Again, the authors refer to Wilson's work, which included two strains of carbon dioxide-dependent "*Brucella melitensis*." These strains also were *melitensis* on the basis of serology, but were *abortus* by all other criteria. The pitfalls of the serological typing of *Brucella* strains have already been discussed in the section on *Brucella*.

Formal exception to the classification of the ram epididymitis bacterium was taken by Meyer & Cameron (106), who based their objection largely on the biochemical behavior of resting cells. One striking point of difference between this and the established brucellae was complete absence of the enzymes involved in the urea cycle in the former, though they are regularly present in all three *Brucella* species. Similarly, the failure of the epididymitis organism to ferment any carbohydrates represents a radical departure from brucellar physiology. Moreover, among workers familiar with both organisms the statement of their cultural and morphological resemblance has by no means been accepted unanimously. One of the most serious objections to inclusion of this bacterium among *Brucella* was the absence, reported by all early investigators, of any serological relationship to this genus. Eventually, Renoux & Mahaffey (126) and Buddle (26) demonstrated cross agglutination between the ram bacterium and various non-smooth strains of *Brucella* species. To what extent these findings should be accepted as bases for classification is open to debate, since *Brucella* has antigens in common with a number of other genera, e.g., *Pasteurella*, *Salmonella*, and *Vibrio* (51). According to Buddle's data the non-smooth *Brucella* were no closer to the epididymitis agents than were smooth members in their biochemical behavior or their dye tolerance. It might be well to keep in mind Schuetze's (131) observation on the "serological cosmopolitanism of rough variants" exhibited by enteric bacteria.

Since there is very real doubt as to the correctness of classifying the ram bacterium as a *Brucella*, the tests designed to establish a particular species of *Brucella*, when applied to this agent, lose much of their relevance. These would include examination for dye tolerance, carbon dioxide requirement, reaction on Renoux's diethyldithiocarbamate medium, duration of hydrogen sulfide production, and even the acriflavine test for smoothness.

Several alternative suggestions have been made regarding classification of the ram epididymitis organism. *Neisseria* has been mentioned (106), but, in this reviewer's opinion, must be rejected on morphological grounds, if on no other. The proposal to include the organism among the pertussis group (87) deserves more serious consideration. Among the similarities are the total inertness toward carbohydrates as determined by conventional fermen-

tation media as well as by the manometric procedures, the patterns of utilization of a great many amino acids (107), the strong catalase activity, and a fastidiousness on primary isolation that diminishes on later passages. The colonial and cellular morphology of the ram epididymitis agent is as close to the *Bordetella* as to the *Brucella* group. No data on serological relations and comparative toxicity are found in the literature. The one real obstacle to including this agent in the genus *Bordetella* is its consistent lack of hemolytic activity, a trait of *Bordetella* species as defined in *Bergey's Manual* (121).

Thus, agreement on the proper classification will probably not be reached for some time, and the ram organism may continue to exist in the uncertain taxonomic haze that has been the abode of the tularemia agent for almost 50 years. Meanwhile, other aspects of the agent and the disease have been explored more fruitfully. The epidemiology of the infection received early attention. Numerous studies in various parts of the world have failed to show any significant reservoir of infection other than the infected ram. Hartley *et al.* (66) demonstrated that the ewe, after breeding exposure to an infected ram, can communicate the infection to other rams mated to her during the same heat period. Ewes did not remain infected from one season to the next, however, and in a recent study (14), still unpublished, the organism could ordinarily be detected in the vagina for only a day following infection, and in exceptional cases for about a week. In no case could susceptible rams be infected by being bred to ewes that had received their exposures more than a month previously. It thus appears that venereal transmission must be prompt, and that, though the spread is reportedly most rapid in the breeding season (66), venereal dissemination from ewe to rams cannot be the only method of spread. Epididymitis occurs regularly in flocks of ram lambs prior to any breeding activity (90). New Zealand (27) workers have suggested rectal copulation among rams to be the agency of dissemination. It has been objected, however, that, although homosexual practices are common among rams and rectal copulation is frequently attempted, completion is rare (90) and it is doubtful that incidental contamination of the fleece and perirectal area is sufficient to establish infection. The possibility of infection from extragenital sources and by non-venereal methods remains to be explored thoroughly. It has been shown that contaminated pastures are not significantly involved in transmission (66), but significant contamination of water and feed supply over short periods has not been ruled out. The fact that many routes of natural exposure, including oral and conjunctival (28), regularly lead to infection (and in most cases to genital involvement), makes it advisable to investigate the possibility of such contamination. In this connection it should also be stressed that feces, urine, saliva, and lacrimal fluids remain as possible sources of infection. Epididymitis is a generalized infection in both sexes, and the causative agent has been recovered for several months following infection from sites remote from the portal of entry, including kidney, ovary, uterus, udder, spleen, and lymph nodes (14).

Isolations from colostrum have also been reported (29). Any role of these foci in the epidemiology of the disease is at present unknown. The effect of their presence on the host appears to be almost nil in both the clinical and pathological sense. Reports by Czechoslovakian (17) investigators of extensive pathology, particularly of the reticuloendothelial system (16), are in conflict with findings by New Zealand (79), and American workers (88); they will require confirmation, particularly since the animals examined were concurrently parasitized by a variety of helminths and sporozoa.

On the basis of reports on the pathology from American and Australasian sources, the organism induces a rather mild and chronic inflammatory response. The gross genital lesions, which are the only macroscopic anatomical manifestation of the disease in rams, are secondary to a low-grade inflammation of epididymal and testicular tissue that results in an encroachment on the lumen of ductules and tubules. When this becomes severe enough to interfere with the normal passage of sperm, stasis and eventually rupture takes place, and sperm enters the interstitial tissue, setting up sperm granulomata and abscesses. These are the palpable lesions of epididymitis. They do not develop in all infected rams by any means. Serological and cultural evidence indicates that only 70 to 80 per cent of infected rams develop gross lesions (13). The remainder excrete the agent in their semen and therefore undoubtedly play an important part in the insidious spread of the disease.

HAEMOPHILUS

Interest in *Haemophilus* in veterinary medicine has been intermittent and uncertain. The genus cannot be located in the index of a most recent text on veterinary microbiology (144). Yet, the last few years have witnessed an increasing number of reports on this group of organisms. *Haemophilus suis*, the oldest representative among the species parasitic on animals, has been extensively investigated as to pathogenicity and antigenic constitution (7, 8). The main object of recent interest was its role in Glässer's disease, a purulent inflammation involving the serous cavities of pigs, and very frequently also the meninges and joints. According to Swedish workers, this disease is caused by *Haemophilus suis*, whereas, in the United States, a *Mycoplasma* is frequently involved (152). Lecce (93) showed that both organisms are capable of causing the syndrome and that a combination of the two was not more pathogenic than either organism alone for pigs deprived of colostrum. We have identified as *Haemophilus* an organism recovered from a condition pathologically indistinguishable from Glässer's disease. This *Haemophilus* differed from *H. suis* in being strongly hemolytic. It required only the codehydrogenase (V factor) as an accessory growth substance. Lecce's organism needed hemin (X factor) as well as DPN as did Lewis and Shope's original isolates of *H. suis*.

The vague connection between *Mycoplasma* and *Haemophilus* in Glässer's

polyserositis is just another point where the paths of these two groups of microorganisms intersect. Of other common properties, the ability of *Haemophilus* and *Mycoplasma* species to accept the Dienes stain (methylene blue-azure II) sets them apart from most bacteria. Both *Haemophilus* and *Mycoplasma* are involved in fowl coryza, a respiratory disease of birds, though their functions in the pathogenesis of the disease are separate (2, 113, 114). *Haemophilus gallinarum* has been shown to be able to cause the condition in its acute form. When pathogenic *Mycoplasma* are superimposed, a more chronic condition results. *Mycoplasma* alone can cause the chronic disease, but the incubation period preceding the onset of symptoms is longer than when *Haemophilus* is present. A still closer relationship between the two organisms has been suggested by McKay and co-workers (100, 101), who published evidence that the *Mycoplasma* associated with coryza are in fact, L forms of *Haemophilus*. By cultivating egg-propagated *Mycoplasmas* on adequate media they succeeded in producing bacteria showing the characteristics of *Haemophilus*. Media-propagated *Mycoplasma* had to be passed through eggs before such conversion could be accomplished. In some cases, conversion was induced through bacteria-free saline *Haemophilus* extracts, in others by *Haemophilus*-derived deoxyribonucleic acids. In all instances—spontaneous, extract-induced, and DNA-induced conversions—egg passage was indispensable. Since other investigators have been consistently unsuccessful in converting *Mycoplasma* strains to bacteria (1), confirmation of these results appears desirable. It would be particularly to the point to establish some kind of relationship—serological, biochemical, or pathogenic—between the *Mycoplasma* and the emerging *Haemophilus* strains.

A final point of contact between *Mycoplasma* and *Haemophilus* that might be mentioned is the reported presence of both or either in cases of atrophic rhinitis of swine (22). This common feature is less impressive since a number of other microorganisms, including trichomonads (139) and *Pasteurella* (61, 62), have also been found in, or even been blamed for, this disease.

Isolation of *Haemophilus* and *Haemophilus*-like organisms from diseased animals has been reported from a great many countries. Diernhofer (47) reported isolation of a *Haemophilus*, which he named *H. citreus*, from the genital tract of cattle affected with various inflammatory processes. We have recovered similar organisms from the mucosa of cows and ewes with vaginitis. Their etiological relationship to the condition with which they were found associated is uncertain, particularly in the case of the ewes that had been experimentally exposed to the ram epididymitis bacterium. Still, their occurrence in this area is interesting in view of recent reports of the isolation of *Haemophilus* from abortions in Montana (55) and Israel (112). The finding of a form of *Haemophilus* in vaginitis in women is of interest in this connection. All strains reported on so far have been hemolytic and, unlike Diernhofer's cultures, required V but not X factor.

We have seen similar kinds of DPN-dependent hemolytic bacteria in cattle, sheep, and hogs, where they were associated with septicemic disease or conditions that can be interpreted as aftermaths of systemic infections, such as arthritis, meningitis, fibrinous pleuritis, and pneumonia. In routine bacteriological tests the various isolates were not identical. One of the pig isolates has been shown to be capable of causing the septicemic disease under experimental conditions. The other strains have not yet been tested in this manner.

Apart from these cultures that could be readily assigned to the genus *Haemophilus* on the basis of their DPN requirement, a different kind of fastidious Gram-negative organism was recovered from disease syndromes whose clinical and pathological features closely resembled those seen in the *Haemophilus* infections (87, 89). For the first representative of this group, the name *Haemophilus agni* was suggested, but certain consistent differences between this and the more classical type of *Haemophilus* have been noted. On the basis of experience with eleven strains of the former and seventeen strains of the latter type, the following table of comparison may be set up (Table I).

TABLE I

COMPARISON OF *HAEMOPHILUS* SP. AND *HAEMOPHILUS*-LIKE ISOLATES FROM ANIMALS

	Haemophilus sp.	"*H. agni*"
Primary culture on infusion media without supplement	–	–
Primary culture on media containing blood or yeast hydrolysate	+	+
Heat stability of growth factor	–	+
Replaceability of growth factor with DPN	+	–
Replaceability of growth factor with hemin	–	–
CO_2 requirement for primary culture	–	+
Adaptability to growth on unsupplemented media	–	+
Hemolysis on beef blood media	+	–

Although the genus *Haemophilus* at present contains organisms that need supplements other than X and V, and even some whose needs are not defined, classification of the group of bacteria typified by *H. agni* must be considered provisional. Its ability to ferment carbohydrates and its lack of hemolytic activity exclude it from *Bordetella* as defined at this time.

The epidemiology of *Haemophilus* infections in animals is relatively unexplored at present. We have encountered them in both sporadic and epidemic form. In the latter instance, serological evidence suggested that infections were far more frequent than clinical disease. A herd or flock out-

break was often preceded by changes in management practices, such as the institution of an intensified feeding program. Until direct experimental data become available, it is not unreasonable to assume that the organisms involved may be part of the normal flora of the mucous membranes, proliferating under conditions of stress.

Conclusion

Research done on the family Brucellacea has been almost as diverse as the members of the group themselves. In surveying the work having some relation to veterinary medicine we have found the length and breadth of experimental objectives, design, and methods amply represented. To attempt to predict what line work in this area will take in the future would be more than presumptuous; it would be foolhardy. For the next few years, it is safe to say, much of it will probably be suggested by the hard facts of disease prevalence and demands for control.

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METABOLISM OF C_1 COMPOUNDS IN AUTOTROPHIC AND HETEROTROPHIC MICROORGANISMS[1,2]

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Introduction

This review discusses the participation of C_1 units, including CO_2, in the microbial synthesis of C_2, C_3, and C_4 compounds, with special reference to the syntheses effected by microorganisms during their growth on such C_1 compounds as sole carbon source. The role of C_1 compounds in the synthesis of larger molecules, such as purines, is not included, nor is the biochemistry of C_1-transfer reactions per se, as it has been adequately reviewed elsewhere (63, 144).

CO_2 as Sole Carbon Source for Growth

The discovery of a cyclic reaction sequence, shown in Figure 1a, which leads to the *de novo* synthesis of a polycarbon organic compound from CO_2, was first made by Calvin and his colleagues in their study of green plant photosynthesis (18). The overall operation of one turn of this cycle results in the coversion of CO_2 to glyceraldehyde-3-phosphate:

$$3CO_2 + 9ATP + 5H_2O + 6TPNH \rightarrow \text{glyceraldehyde-3-P} + 9ADP + 6TPN + 8Pi \quad \text{(i)}$$

The cycle contains only two enzymes that are, as far as is known, unique to it namely phosphoribulokinase (64, 67) and carboxydismutase (143, 199) catalyzing reactions (ii) and (iii), respectively:

$$\text{Ribulose-5-phosphate} + ATP \rightarrow RuDP + ADP \quad \text{(ii)}$$

$$\begin{array}{lcl} CH_2OPO_3H_2 & & CO_2H \\ | & & | \\ C{=}O + CO_2 + H_2O & \rightarrow & 2CHOH \\ | & & | \\ (CHOH)_2 & & CH_2OPO_3H_2 \\ | & & \\ CH_2OPO_3H_2 & & \\ \text{RuDP} & & \text{PGA} \end{array} \quad \text{(iii)}$$

[1] The survey of the literature pertaining to this review was concluded in November 1960.

[2] The following abbreviations will be used: ADP and ATP (adenosine di- and triphosphates); CoA (coenzyme A); GDP and GTP (guanosine di- and triphosphates); IDP and ITP (inosine di- and triphosphates); PGA (3-phosphoglyceric acid); P_i, (orthophosphate); RuDP (ribulose-1,5-diphosphate); THF (tetrahydrofolic acid); TPN and TPNH (oxidized and reduced triphosphopyridine nucleotide); TPP (thiamin pyrophosphate).

[3] I wish to record my indebtedness to Prof. Sir Hans Krebs, F. R. S., and to Drs. H. L. Kornberg, J. Lascelles, and M. F. Utter for their helpful criticisms and advice.

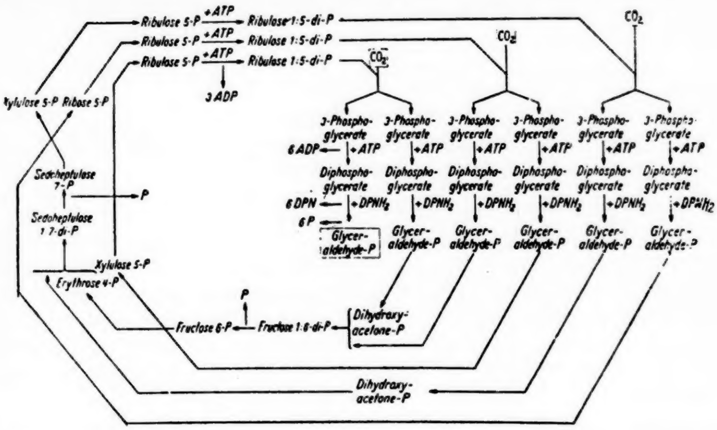


FIG. 1a. [Taken from Krebs, H. A., and Kornberg, H. L., *Ergebnisse der Physiologie*, **49**, 248 (1957).]

There is still uncertainty as to the relative importance *in vivo* of a variant on the cycle, shown in Figure 1b, which was proposed by Racker (146) who demonstrated its operation with enzymes obtained from spinach. The latter cycle differs slightly from that shown in Figure 1a in the reactions which lead to the formation of the three pentose phosphate molecules from

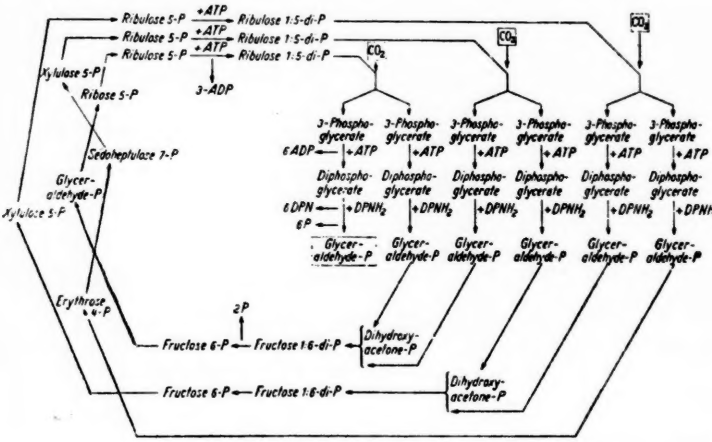


FIG. 1b. [Taken from, Krebs, H. A., and Kornberg, H. L., *Ergebnisse der Physiologie*, **49**, 245 (1957).]

the five triose phosphate molecules. This difference results in the involvement of transaldolase, but not sedoheptulose-1,7-diphosphate, in Racker's variant of the cycle. Unless specified, however, both sequences will be referred to as the ribulose diphosphate cycle of CO_2 fixation, or RuDP cycle.

That the RuDP cycle is the major pathway by which CO_2 is fixed by green plants is shown by the recent work of Bassham & Kirk (17), who measured the rates of appearance of ^{14}C in individual compounds formed by *Chlorella pyrenoidosa* during steady-state photosynthesis. These rates were compared with the rates of $^{14}CO_2$ and CO_2 disappearance from the gas phase during the same experiments. The authors showed that the compounds formed from the carbon reduction cycle via carboxylation of RuDP account for at least 70 to 85 per cent of the total CO_2 uptake. They also showed that the carboxylation of RuDP *in vivo* during photosynthesis appears to be followed by conversion of the product to one molecule of PGA containing the newly incorporated $^{14}CO_2$, and one molecule of some other C_3 compound. This latter compound has not been identified, and Bassham & Kirk suggested that it might be either a small pool of PGA separated by some form of compartmentation from the principal pool, or triose phosphate resulting from a reductive cleavage of the carboxylation product of RuDP:

$$\begin{array}{llll}
CH_2OPO_3H_2 & & CO_2H & \quad CHO \\
| & & | & \quad | \\
C{=}O + CO_2 + 2H & \rightarrow & CHOH & + \; CHOH \\
| & & | & \quad | \\
(CHOH)_2 & & CH_2OPO_3H_2 & \quad CH_2OPO_3H_2 \\
| & & & \\
CH_2OPO_3H_2 & & & \quad \text{Triose phosphate}
\end{array} \qquad \text{(iv)}$$

This reaction has always been emphasized by Calvin and his colleagues (18) as an alternative to the hydrolytic cleavage (iii).

The possibility, raised by Racker (147), that the *in vivo* carboxylation of RuDP is in some way different from the hydrolytic carboxylation observed with the purified enzyme, still remains open. Racker pointed out that

> . . . some of the properties of RuDP carboxylase, such as its low activity and its low affinity for bicarbonate do not appear compatible with the known rate of photosynthesis at low carbon dioxide concentrations. On the other hand, the experiments of Calvin and his collaborators with $^{14}CO_2$ indicate so strongly the operation of the cycle *in vivo* that one is tempted to suspect that a link in the enzymic chain is still missing. . . .

It must be emphasized that the uncertainties outlined above concern details of mechanism, and do not affect the overall validity of the RuDP cycle, which is now generally accepted [For reviews see (15, 16, 62, 197)].

The evidence for occurrence of the RuDP cycle in autotrophic organisms other than green plants will now be examined.

Non-photosynthetic organisms.—Badin & Calvin (5) and Glover, Kamen & van Genderen (57) carried out a radioautographic analysis of the path of $^{14}CO_2$ into cellular metabolites of *Scenedesmus* D_3 and *Rhodospirillum rubrum*,

respectively, while these organisms were oxidizing hydrogen aerobically in the dark. It was shown that, as in the case of photosynthesizing algae, early labelling of phosphorylated compounds, especially PGA, occurred. Although the RuDP cycle had not at that time been elucidated, the above findings constituted presumptive evidence that a process of CO_2 fixation similar to that operating in green plants, could occur during non-photosynthetic CO_2 fixation.

A rigorous establishment of the occurrence of the RuDP cycle in the non-photosynthetic autotroph, *Thiobacillus denitrificans*, was made shortly after the elucidation of the cycle in plant photosynthesis. To establish the cycle with the same degree of thoroughness as in green plants, it was necessary to satisfy the following requirements: (*a*) An analysis of the metabolites labelled during assimilation of $^{14}CO_2$ must show PGA to be the first labelled compound. Other intermediates of the cycle must also become labelled at early times (4). (*b*) The labelling patterns of intermediates of the cycle must conform with those predicted by operation of the cycle (111). (*c*) The pool sizes of the acceptor compound for CO_2, viz., RuDP, and the product of the carboxylation, viz., PGA, must show the same dependence on the sudden deprivation of reducing power or of CO_2 as was observed in similar experiments with photosynthetic organisms (111). (*d*) All the enzymes necessary for the operation of the cycle must be present in the organism (186, 187).

The appended references indicate the work carried out on *T. denitrificans* which satisfied each particular criterion. The results of all of these experiments were in excellent agreement with those predicted for the RuDP cycle, and establish this cycle as the principal mode of CO_2 fixation operating in *T. denitrificans*. Evidence of varying degrees of thoroughness has since been used to establish the occurrence of the cycle in several other autotrophically-grown organisms (Table I).

In view of the rapid incorporation of $^{14}CO_2$ into PGA and sugar phosphates observed in autotrophically-grown *Hydrogenomonas facilis* (24, 108, 123), the finding of formic acid and acetic acid (doubly-labelled) among the earliest labelled compounds by Koffler and his co-workers (122, 123), is unexpected. More details are needed to assess the significance of this, especially as Bergmann, Towne & Burris (24), in similar experiments, were not able to detect significant incorporation of tracer into any volatile acids.

Carboxydismutase has been implicated in pentose metabolism by the report (51) that *Escherichia coli* grown aerobically on xylose or arabinose contains this enzyme; its possible function is, however, obscure. In the opinion of the reviewer, the experimental data are, as yet, inadequate to prove the presence of the enzyme in this organism. The evidence consists of the finding that $^{14}CO_2$ fixation by crude extracts of the organism is increased fourfold on the addition of RuDP. No formation of labelled PGA was detected in the absence of RuDP; in its presence, a small quantity of radioactive PGA was found, amounting to 7 per cent of the total ^{14}C fixed. There is no check, however, to prove that the labelled PGA did not, in fact,

TABLE I

SOME AUTOTROPHIC MICROORGANISMS IN WHICH EVIDENCE FOR THE OCCURRENCE OF THE RuDP CYCLE HAS BEEN OBTAINED

Type of organism	Name of organism	Reference
Non-photosynthetic bacteria	*Hydrogenomonas facilis*	(24, 108)
	Hydrogenomonas ruhlandii	(153)
	Hydrogenomonas spp.	(196)
	Micrococcus denitrificans	(84)
	Thiobacillus denitrificans	(4, 111, 186, 187)
	Thiobacillus thiooxidans	(177, 179)
	Thiobacillus thioparus	(67, 152)
	Nitrobacter agilis	(103)
Photosynthetic bacteria metabolizing CO_2 in dark aerobically with inorganic electron donor	*Scenedesmus* D_3	(5)
	Rhodospirillum rubrum	(57)
	Rhodopseudomonas capsulatus	(170)
Photosynthetic bacteria photometabolizing CO_2 anaerobically with inorganic electron donor	*Rhodospirillum rubrum*	(57)
	Rhodopseudomonas capsulatus	(170)
	Chromatium	(51, 52, 99, 196)

come from the remaining 93 per cent of the total isotopic material formed, the nature of which is not given.

Wellerson and co-workers have suggested (201) carboxylation of RuDP as an explanation for the labelling pattern observed in lactic acid obtained from the protozoon *Trichonomonas vaginalis*. This organism, when grown anaerobically in complex medium, excretes lactic and malic acids into the medium. If the incubation is carried out in the presence of $^{14}CO_2$ the lactate is labelled only in the carboxyl group, while the malate is non-radioactive. This latter finding makes it unlikely that the $^{14}CO_2$ finds its way into lactate via fixation into malate and oxalacetate:

$$\begin{array}{l} CO_2 + \text{pyruvate (or phosphopyruvate)} \rightleftharpoons \text{malate or oxalacetate} \rightleftharpoons \text{fumarate} \\ \qquad\qquad \upharpoonleft\downharpoonright \\ \qquad\quad \text{lactate} \end{array} \tag{v}$$

The organism is devoid of carboxylase activity, eliminating the reaction:

$$CH_3\cdot CO\cdot CO_2H \rightleftharpoons CH_3\cdot CHO + CO_2 \tag{vi}$$

and the authors suggest that the labelling pattern may arise by carboxylation of RuDP; however, extracts of the organism were not tested for carboxydismutase. It would seem more likely that the labelling pattern arises from an exchange reaction between pyruvate and CO_2, similar to that commonly encountered in anaerobic organisms.

A comprehensive series of reviews has recently appeared dealing respec-

tively with autotrophy in general (92), iron bacteria (154), nitrifying bacteria (47), sulphur and selenium bacteria (188), and hydrogen-oxidizing bacteria (155).

Photosynthetic bacteria.—The following work indicates that the main path of carbon assimilation in bacterial photosynthesis, where CO_2 is the only carbon source, is the same as in green plant photosynthesis. Compared with the many detailed experiments that have established, with varying degrees of thoroughness, the pathway in the chemosynthetic autotrophs, the experimental evidence in the case of the photosynthetic bacteria is surprisingly limited. Radioautographic studies of the pathway of assimilation of $^{14}CO_2$ by *Rhodopseudomonas capsulatus* (170) and *R. rubrum* (57) under anaerobic conditions in the light, and in the presence of inorganic electron donors, showed rapid incorporation of isotope into PGA, while extracts of *Chromatium* grown autotrophically in the light have been shown to contain carboxydismutase (51, 52, 99, 196).

All of the available evidence reported so far shows that organisms that use CO_2 as sole carbon source incorporate the bulk of it by the RuDP cycle regardless of their energy source. No other sequence of reactions has been found which results in the total synthesis from CO_2 of an organic compound containing more than one carbon atom. Hence, it is unambiguous at present to regard the RuDP cycle of CO_2 fixation as being synonymous with autotrophic CO_2 fixation.

The Role of CO_2 in Photometabolism of Organic Substrates

The incorporation of carbon by photosynthetic bacteria growing on CO_2 as sole carbon source has been dealt with in the previous section; more complex problems are presented when both organic substrate and CO_2 are incorporated during photosynthetic growth. The following discussion is limited to aspects of the role of CO_2 in such bacterial photosynthesis; more comprehensive reviews on general photometabolism have been written by Gest & Kamen (54) and by Elsden (46).

In bacterial photosynthesis, organic substrates have been viewed in the past with emphasis on their role as sources of reducing power for the reduction of CO_2, rather than on their role as sources of cellular carbon. This emphasis has now shifted, largely because of two important developments: (*a*) The demonstration, presented mainly by Arnon and his colleagues (2, 3). that the function of light in photosynthesis is not to photolyse water as van Niel proposed (118), but to provide ATP by photosynthetic phosphorylation and to raise electrons derived from hydrogen donors (organic or inorganic) to higher reducing potentials. (*b*) The finding of Stanier and his colleagues that

> . . . the main function of organic substrates in bacterial photosynthesis is to serve as readily assimilable sources of carbon. They also provide reducing power for CO_2 fixation, as postulated by van Niel (118); but the fixation of carbon dioxide, although qualitatively important for the growth of purple bacteria with organic substrates, is usually of minor quantitative importance as a source of cellular carbon. (168)

Stanier *et al.* studied the photometabolism of various substrates by *R. rubrum*, and found that when the rate of uptake of the carbon substrate exceeded that of cellular synthesis, the assimilated carbon was stored either as poly-β-hydroxybutyrate or a glycogen-like polysaccharide. The nature of the carbon source determined which of these polymers was formed predominantly. Those substrates which could be converted directly to acetyl units without the intermediate formation of pyruvate yielded mostly poly-β-hydroxybutyrate. Assimilation of CO_2, or substrates such as succinate which could be directly converted to pyruvate with generation of reducing power, yielded mostly polysaccharide. The difference between the oxidation levels of the substrate and assimilated product resulted in net evolution of CO_2, where the substrate was more oxidized than the product, as with acetate:

$$\underset{\text{acetic acid}}{nC_2H_4O_2} + 2nH_2O \rightarrow 2nCO_2 + 8nH \qquad \text{(vii)}$$

$$8nC_2H_4O_2 + 8nH \rightarrow \underset{\text{poly-}\beta\text{-hydroxybutryic acid}}{4(C_4H_6O_2)_n} + 8nH_2O \qquad \text{(viii)}$$

$$\text{Sum: } 9nC_2H_4O_2 \rightarrow 4(C_4H_6O_2)_n + 2nCO_2 + 6nH_2O \qquad \text{(ix)}$$

or by fixation of CO_2, where there was an excess of reducing power, as with butyrate:

$$\underset{\text{butyric acid}}{2nC_4H_8O_2} \rightarrow \underset{\text{poly-}\beta\text{-hydroxybutyric acid}}{2(C_4H_6O_2)_n} + 4nH \qquad \text{(x)}$$

$$4nH + nCO_2 \rightarrow \underset{\text{polysaccharide}}{(CH_2O)_n} + nH_2O \qquad \text{(xi)}$$

$$\text{Sum: } 2nC_4H_8O_2 + nCO_2 \rightarrow 2(C_4H_6O_2)_n + (CH_2O)_n + nH_2O \qquad \text{(xii)}$$

The concept of different substrates being assimilated to storage products at different oxidation levels elucidates several previously puzzling facts. It was observed by Ormerod (124) that illuminated anaerobic suspensions of *R. rubrum* gave a net output of CO_2 in the presence of pyruvate, lactate, succinate, fumarate, and malate, and a net fixation of CO_2 in the presence of propionate and butyrate. Each of these substrates caused an increase in the incorporation of $^{14}CO_2$ over and above that found in the control without substrate. Acetate, however, which is at the oxidation level of cellular material, evolved CO_2 and actually reduced the incorporation of $^{14}CO_2$ over the control. Similar behaviour had been noted by Glover & Kamen (56) who observed that the presence of acetate diminished the incorporation of $^{14}CO_2$ by *R. rubrum* in the presence of inorganic electron donors such as H_2. These observations were not in accord with the idea of organic substrates functioning as reductants of CO_2. However, since the assimilation of acetate into poly-β-hydroxybutyrate occurs in non-growing cultures of *R. rubrum*, the decrease in $^{14}CO_2$ fixation resulting from the addition of acetate to such bacterial suspensions can now be explained in terms of the " . . . conversion of acetate to poly-β-hydroxybutyrate competing with CO_2 fixation for the limited reducing power available . . . " (168). Stanier and his colleagues were further able to show that conversion of the polymer to carbohydrate and

nitrogenous cellular constituents depended on the presence and fixation of CO_2. Thus, a non-growing suspension of *R. rubrum* may synthesize polymer from acetate with little CO_2 incorporation, whereas a suspension growing on acetate, where general cellular biosynthesis is taking place, may incorporate substantial amounts of CO_2.

These newer concepts of the direct utilization of organic substrates for photosynthetic growth have been extended to the green sulphur bacterium *Chlorobium limicola* by Sadler & Stanier (149). It had previously been shown (91, 117) that species of *Chlorobium* were strictly anaerobic, photosynthetic autotrophs which used inorganic sulphur compounds as electron donors. In many respects, their biochemistry seemed very similar to purple sulphur bacteria except that they appeared unable to utilize organic compounds for growth. Sadler & Stanier have now shown that the presence in the growth medium of several organic compounds, especially acetate, caused a detectable increase in growth. It was found that under these conditions acetate could serve as a general source of cellular carbon. Its utilization, however, was strictly proportional to the concentrations of both CO_2 and H_2S (the electron donor). The authors suggested that this double dependence may result from an inability of the organism to oxidize acetate, and, unlike *R. rubrum*, to effect reaction (vii). Hence, *C. limicola* would be unable to derive from acetate the reducing power and CO_2 essential for the synthesis of cell constituents.

The photometabolism of acetate in the obligatory anaerobic phototroph, *Chromatium*, has been the subject of recent study. It has been noted that the carboxydismutase activity falls when the metabolism of the organism is changed from autotrophic growth with inorganic electron donors, to growth on acetate (52, 196). This is consistent with the findings of Losada *et al.* (99) who report that the addition of acetate to a suspension of *Chromatium* causes no stimulation of $^{14}CO_2$ incorporation. Fuller & Kornberg showed (52) that a key enzyme of the glyoxylate cycle (79), *iso*-citratase, was adaptively formed when the carbon source was changed from CO_2 to acetate, and hence suggest that the organism uses this cycle in the synthesis of cell constituents from acetate. Such a cycle would provide reducing power, while ATP is synthesized via photosynthetic phosphorylation.

In those photosynthetic bacteria where CO_2 fixation does take place during assimilation of organic substrates, there is evidence that the RuDP cycle operates, although much remains unknown regarding the balance that is struck between the operation of the cycle and the incorporation of the organic substrate. Glover *et al.* (57) showed that $^{14}CO_2$ was incorporated mainly into PGA during photometabolism of acetate by *R. rubrum*, and Lascelles has demonstrated (93) the formation of carboxydismutase in *Rhodopseudomonas spheroides* and *Rhodopseudomonas palustris* growing anaerobically in the light on either a malate-glutamate medium, or a butyrate-$NaHCO_3$ medium. The involvement of carboxydismutase only in the anaerobic-light metabolism of these organisms is very clearly shown in this work, since the adaptive formation of the enzyme is shown to be de-

pendent on simultaneous anaerobiosis and illumination. Introduction of oxygen or cessation of illumination resulted in cessation of carboxydismutase synthesis by the growing organisms. It is suggested that the major physiological role of the RuDP cycle in such conditions is the provision of a sink for excess reducing power generated by the oxidation of the carbon substrate. Further evidence for the operation of the RuDP cycle during growth on organic substrates by *R. spheroides* and *R. rubrum* has been obtained by Elsden (45, 46), who has studied the effect of cyanide ($10^{-3}M$) on the photometabolism of several substrates. He has found that in the case of *R. rubrum* the photometabolism of succinate, propionate, and butyrate is about 80 per cent inhibited; lactate, malate, and fumarate about 50 per cent and acetate only 20 per cent inhibited. The pattern with *R. spheroides* is similar, except that in the case of acetate, cyanide was slightly stimulatory. Comparing these results with those of Ormerod (124), it appears that the greater the amount of CO_2 assimilated per mole of substrate the greater the sensitivity to cyanide. If the cyanide sensitivity is caused by carboxydismutase inhibition, which seems likely from work with chloroplasts (185), then the experiments may demonstrate the part played by the RuDP cycle in the photometabolism of these substrates.

There is a puzzling feature about the involvement of CO_2 in acetate photometabolism for which no explanation has yet been found. Cutinelli *et al.* (37) found that in the alanine obtained from *R. rubrum* grown photosynthetically on acetate and bicarbonate, the labelling pattern indicated that the carboxyl group of alanine was derived almost exclusively from CO_2, while the α- and β-carbon atoms originated from the carboxyl and methyl groups of acetate, respectively. These data strongly suggested that a carboxylation of a C_2 unit derived directly from acetate had taken place. The nature of this proposed C_2+C_1 condensation remains unknown. Neither is it known why the labelling pattern expected in PGA from the fixation of $^{14}CO_2$ via the RuDP cycle, was not found in the carbon skeleton of alanine. The reason for this unexplained labelling may possibly underlie the unknown role of CO_2 in the mobilization of the poly-β-hydroxybutyrate polymer during glycogen synthesis. Stanier *et al.* (168) point out that in this respect it appears as though CO_2 plays some essential role in the formation of C_3 compounds from acetate. This question will be referred to later in the review, as a similar labelling pattern has been found in alanine obtained from *Clostridium kluyveri* grown on acetate, ethanol, and CO_2 (182).

Organic C_1 Compounds as Sole Carbon and Energy Source

Many species of bacteria capable of growth on C_1 compounds as sole carbon and energy source have been characterised and studied; the better authenticated of these are listed in Table II. The means whereby such organisms grow has long been the subject of speculation. It has been suggested (25, 95, 119) that they might couple the energy of oxidation of the organic substrate to the reduction of CO_2 and the synthesis therefrom of all

TABLE II

SOME MICROORGANISMS WHICH CAN GROW ON C_1 COMPOUNDS AS SOLE SOURCE OF CARBON AND ENERGY

Name	Properties	References to description of organism
Aerobes:		
*Bacillus extorquens** (*Pseudomonas extorquens, Vibrio extorquens*)	Pink coloured. Grows on variety of compounds including methanol, formate, and formaldehyde.	(14)
Bacillus sphaericus	Grows on N-methyl urea.	(66)
Hyphomicrobium vulgare	Grows on methanol, formate.	(110, 175)
*Methanomonas methanica** (originally called *Bacillus methanicus*, see *Pseudomonas methanica*)	Pink coloured. Grows on methane.	(23, 163)
Methanomonas methanooxidans	Grows on methane.	(28, 171)
*Protaminobacter ruber**	Pink coloured. Grows on many substrates including methanol, methylamine, and formate.	(23, 39, 40, 90)
Pseudomonas AM1*	Pink coloured. Grows on many substrates including methanol, methylamine and formate.	(139, 140)
Pseudomonas amino vorans (Seven different strains)	Grows on various substrates including methanol, methylamine, formate, and formamide.	(39, 40)
*Pseudomonas methanica** (27 strains isolated, the authors claim that one of the strains is identical with *Methanomonas methanica* and suggest the latter name be changed to *P. methanica* [(Söhngen) nov. comb.]	Pink, yellow, brown, and colourless strains isolated. All strains will grow only on methane or methanol.	(42, 61, 94)
Pseudomonas oxalaticus (several strains)	Grows on many substrates including formate.	(75)
Pseudomonas PRL-W4*	Pink coloured. Grows on several substrates including methanol. Biotin stimulates growth on latter substrate.	(71)
Vibrio oxaliticus	Growth on several substrates including formate in presence of yeast extract.	(25)
Not named	Grows on methane.	(65)
Anaerobes:		
Methanobacterium formicicum	Ferments formate only, CO in very low concentrations.	(78, 156)
Methanococcus vannielli	Ferments formate only.	(167)
Methanosarcina barkerii	Ferments acetate, methanol, CO.	(156)

* These organisms show so many similarities to strains of *Pseudomonas methanica* that a reclassification of them may be appropriate.

cell constituents. This would then be a variant of chemosynthetic autotrophy in which the organic compound would merely play a role similar to the inorganic electron donor. The occurrence of this autotrophic type of metabolism has now been established by Quayle & Keech (132, 133, 134) in *Pseudomonas oxalaticus* grown on formate as sole carbon and energy source. The experimental findings can be summarised as follows: (*a*) The specific radio-

activity of cellular material obtained from cells grown on ^{14}C formate in air was reduced 17-fold by bubbling air—CO_2 (95:5, v/v) through the growing culture. Thus, at least 94 per cent of the carbon incorporated must have passed through the stage of CO_2, or a compound in ready equilibrium with it (133). (*b*) Isotope from ^{14}C formate or ^{14}C bicarbonate appeared most rapidly in PGA and phosphates of glucose, fructose, sedoheptulose, and ribose; the only other labelled compound seen at the earliest times was malic acid in relatively small amounts (132, 133). (*c*) Cell-free extracts of the organism contain the two enzymes unique to the RuDP cycle, carboxydismutase and phosphoribulokinase (134). The involvement of these enzymes in formate metabolism is shown by the finding that the enzymes are under adaptive control, and are not present in *P. oxalaticus* grown on any substrate tested other than formate (135, 137). No evidence was found for a carboxydismutase-catalyzed incorporation of any C_1 unit other than CO_2 into PGA. The reaction between formate and RuDP giving PGA, which was catalyzed by crude cell-free extracts, was found to be due to a preliminary oxidation of the formate to CO_2 by a particulate fraction of the extract which could be removed by centrifugation (134). All of the evidence points to growth on formate by *P. oxalaticus* as a strictly autotrophic process, the bulk of the carbon being fixed in the form of CO_2 by a pathway similar to that in photosynthetic and chemosynthetic organisms, the necessary energy being derived from oxidation of formate.

In contrast to growth on formate, it has been shown that growth on oxalate by *P. oxalaticus* involves a completely different pathway. Neither carboxydismutase nor phosphoribulokinase are synthesized by the organism growing on oxalate (135, 137). The primary steps in its assimilation have been shown (136, 141, 142) to consist of activation to oxalyl coenzyme A, followed by reduction to glyoxylate by TPNH. The path of glyoxylate then follows that elucidated by Kornberg & Gotto (81, 82) in cells grown on other substrates, e.g., glycollate, the principal catabolic product of which is glyoxylate. Hence, although oxalate is at a slightly higher oxidation level than formate, its C—C bond is preserved for biosynthetic purposes.

An interesting question is raised in the adaptation of the cells from growth on formate to growth on oxalate. This adaptation involves a lag period of no growth which extends up to 15 hr. It would seem that the synthesis of the enzymes which accomplish the decarboxylation of oxalate to formate might enable the cell to use its existing enzymic apparatus temporarily to incorporate CO_2 by the RuDP pathway in an oxalate growth medium, before control mechanisms stopped the synthesis of key enzymes of this cycle, in order to deflect the oxalate carbon into the more favoured assimilation pathway. However, cells harvested at this particular stage appeared to fix only small quantities of $^{14}CO_2$ compared with the formate-grown organisms, showing that despite the availability both of CO_2 and the enzymes necessary to incorporate it by the RuDP cycle, the operation of the cycle is inhibited.

More data are needed before the extent of occurrence of the RuDP cycle in general microbial growth on organic C_1 compounds can be assessed. To date, *P. oxalaticus* is the only organism growing on such compounds in which the operation of the RuDP cycle has been established. It might be expected, especially with a C_1 substrate more reduced than formate, that rather than synthesizing all cellular material by reduction of CO_2 it would be more economical to conserve the reduction level of such a substrate by at least some direct assimilation of the carbon as reduced C_1 units. There is evidence, which will now be examined in detail, that a heterotrophic metabolism of this type does operate in some microorganisms.

Pseudomonas methanica.—Foster and co-workers have isolated some thirty types of bacteria which grow aerobically on methane as sole carbon and energy source (42, 94). All of the organisms are obligatorily dependent on methane or methanol for growth, being unable to utilize a great many other conventional hydrocarbon or non-hydrocarbon substrates. It is considered (94) that these bacteria belong to one species, comprising four varieties based on pigmentation: *Pseudomonas methanica* (Söhngen), pink; *P. methanica* var. *fulva*, yellow; *P. methanica* var. *fusca*, brown; *P. methanica* var. *incolorata*, non-pigmented.

The metabolism of many compounds by these species has been shown to vary depending on whether the cells are "resting" or growing (94). "Resting" cells oxidized methanol, ethanol, *n*-propanol, *n*-butanol and *n*-pentanol to the corresponding carboxylic acids, and formic acid was also oxidized. A wide range of amino acids, carboxylic acids, and sugars were not oxidized. Growing cells, however, oxidized several labelled amino acids and glucose to $^{14}CO_2$, and incorporated ^{14}C into many amino acid components of the bacterial protein. Some of the labelled amino acids appeared to have been incorporated unchanged into the protein.

Determinations were made of the specific radioactivity of cell carbon resulting from growth of the bacteria on unlabelled methane in the presence of $^{14}CO_2$, and these were compared with similar determinations carried out on the typical heterotrophs, *E. coli* and *Pseudomonas fluorescens*, growing on glucose in the presence of $^{14}CO_2$. Leadbetter & Foster found, in the case of *P. methanica*, that the specific activity of the cellular carbon varied from 30 to 70 per cent of the specific activity of the exogenous $^{14}CO_2$, the figures varying within these limits with variously pigmented strains. Figures of 20 to 33 per cent were obtained with the *E. coli* and *P. fluorescens* growing on glucose in the presence of $^{14}CO_2$. The figures are almost certainly too high, for, as the authors point out, the value used for the specific radioactivity of the $^{14}CO_2$ is that obtained at the end of the experiment, when it will be considerably less than at the beginning. Nevertheless, the data indicate the operation of metabolic pathway(s) during growth of these bacteria on methane in which all the carbon incorporated by the cell does not pass through a stage readily exchangeable with CO_2. In some cases, no more cell carbon had exchanged with CO_2 than that observed during growth of typical

heterotrophs on glucose. In other words, during growth on methane, a heterotropic assimilation of reduced C_1 units may take place, in contrast to the purely autotrophic pathway found in *P. oxalaticus* growing on formate.

Pseudomonas PRL-W4.—Kaneda & Roxburgh have isolated (71) a red-pigmented soil organism, *Pseudomonas* PRL-W4, which is capable of growth on many substrates including methanol, but excluding methane, formate, or formaldehyde. Biotin was shown to be a growth factor necessary for growth on methanol. The organism resembles, in many characteristics, *P. methanica* isolated by Dworkin & Foster, but differs in some respects such as nutritional requirements. Kaneda & Roxburgh (70) studied the incorporation of ^{14}C methanol and ^{14}C bicarbonate into methanol-grown *Pseudomonas* PRL-W4 and found that in both cases the first labelled compound to be formed was serine. No labelled phosphorylated compounds were detected, showing that the RuDP cycle could not be operating. A series of isotopic experiments was performed (72), mainly with resting cell suspensions of *Pseudomonas* PRL-W4, to test the effect of the presence of various combinations of methanol, formaldehyde, formate, and carbonate on fixation of either ^{14}C methanol or $^{14}CO_2$ by the cells. On the basis of these experiments, Kaneda & Roxburgh suggest that two simultaneous pathways for the fixation of C_1 compounds exist: carbonate and formate are involved in one pathway, and methanol and formaldehyde in the other. It is presumed that both the formate and formaldehyde are incorporated into serine, possibly by a serine hydroxymethylase system.

Pseudomonas AM1.—A pink-coloured organism, *Pseudomonas* AM1, has been isolated by Quayle & Peel (139, 140) that is similar to *P. methanica* and *Pseudomonas* PRL-W4, but differs in several characteristics, especially in the range of substrates which will support growth. It grows on many compounds, including methylamine, methanol, and formate, but excluding methane. No cofactor requirement for growth has been detected. The path of carbon assimilation in the methanol-grown organism has been studied by following the incorporation of ^{14}C methanol and ^{14}C bicarbonate into the cells by chromatography and radioautography (138). Using either tracer, a rather complex pattern of incorporation of radioactivity appeared, even at the earliest times of incubation. In both cases, however, the pattern obtained was very similar. The radioactivity mainly appeared in malate and amino acids at early times; labelled phosphorylated compounds, citrate and polysaccharide appeared later. The radioactivity initially incorporated into the amino acid fraction from ^{14}C methanol was mainly in serine, as found by Kaneda & Roxburgh (70, 72), whereas that from ^{14}C bicarbonate appeared in glycine and serine in a ratio of 6:4, respectively. (The ratio is calculated from the total counts on the chromatograms in each of these compounds.) This kinetic analysis, together with the fact that no carboxydismutase was found in cell-free extracts of the organism, shows that the RuDP cycle does not operate. The specific radioactivity of cellular material obtained from cells grown on ^{14}C methanol in air was

reduced by half on bubbling air—CO_2 (99:1, v/v) through the growing culture, showing that at least 50 per cent of the methanol carbon passes through a stage which is exchangeable with CO_2.

These results suggest that growth on methanol may involve CO_2 fixation into a C_2 compound and condensation of the C_2 compound with a C_1 unit, derived from methanol, to give a C_3 compound. The kinetic experiments indicate that glycine and serine may be these C_2 and C_3 compounds, although they may be only reflections of undetected primary intermediates such as glyoxylate and hydroxypyruvate. Support for the idea of a primary carboxylation giving glycine, or a closely related compound, is given by the finding that the uniform labelling of glycine obtained from protein hydrolysates of cells grown on ^{14}C methanol in air, is changed to a pattern in which only 3 per cent of the label in glycine is in the carboxyl-carbon when the growing culture is continually flushed with air—CO_2 (99:1, v/v). Whether the C_2 compound is formed by a direct C_1+CO_2 condensation, or is the result of a cyclic series of reactions, is not yet known.

Results with formate-grown *Pseudomonas* AM1 indicate an essentially similar pattern, the only difference probably being a reduction of formate in the form of a tetrahydrofolate (138).

Hyphomicrobium vulgare.—A considerable amount of work has been done (76, 85, 110, 114, 115) on this peculiar stalked bacterium which is able to grow on methanol or formate as sole carbon source, but its very slow pellicular growth is a severe handicap to investigation of its metabolism. Näveke & Engel (115) have summarised most of the work published on this organism and, while stating that it is not possible to decide whether or not the bulk of its carbon is fixed in the form of CO_2, they list properties which they consider to be indirect evidence supporting the classification of the organism as an autotroph: (*a*) Slow growth (110). (*b*) No growth on glucose, sucrose, mannose, or raffinose (110). (*c*) The presence of $^{14}CO_2$ during growth leads to radioactivity appearing in amino acids isolated from the organism (110). (*d*) The organism oxidizes formate and oxalate to CO_2 and water and releases 85 to 95 per cent of the energy as heat (85). Since the nitrifying bacteria oxidize inorganic nitrogen compounds with the release of 93 to 95 per cent of the available energy as heat, the authors state that the similarity in the case of formate or oxalate oxidation by *H. vulgare* leads to the great probability that these substrates serve only as energy sources and not as carbon sources.

In the opinion of the reviewer, the difficulties that attend both the measurement of the efficiency of energy utilization in autotrophic and heterotrophic bacteria and the interpretation of the experimental results are such [see Larsen (92)], as to make most uncertain any assignment of mode of metabolism based on such values.

Recent evidence of a more direct nature indicates that the metabolism of methanol by *H. vulgare* is not autotrophic, but is probably similar to the type of metabolism encountered in *Pseudomonas* PRL-W4 and *Pseudomonas*

AM1. It has been found (131) that extracts of methanol-grown *H. vulgare* do not contain carboxydismutase, and the pattern of incorporation of ^{14}C methanol and $^{14}CO_2$ into cell constituents is almost identical with that found in methanol- or formate-grown *Pseudomonas* AM1.

The results of the work done on the four microorganisms described above allow the following generalisations to be made as to the synthesis of cell constituents by bacteria growing on organic C_1 compounds: (*a*) An autotrophic type of metabolism has been found in one organism (*P. oxalaticus*), growing on formate. (*b*) Evidence for a heterotrophic type of metabolism, involving the concomitant assimilation of substantial quantities of both CO_2 and C_1 units more reduced than CO_2, has been found in four microorganisms (*P. methanica*, *Pseudomonas* PRL-W4, *Pseudomonas* AM1, and *H. vulgare*) growing on methane, methanol, or formate. The evidence, so far, indicates that an essentially similar pathway may operate during growth of the last three organisms on methanol, and, in the case of *Pseudomonas* AM1, on formate as well. The necessary isotopic experiments have not yet been carried out on growth on methane to see if this substrate also involves the suggested common pathway.

These facts raise the question of the use of the terms "heterotroph" and "autotroph" in such cases. The problem seems to have suffered much unnecessary confusion in the past, owing to the opinion held by many authors that microorganisms growing on C_1 compounds are autotrophic, without any evidence to indicate if this is indeed so. This has been well expressed by Foster (50).

> It is more the fact that these organisms synthesize their complex cell constituents from simple 1-carbon compounds chemically analogous to carbon dioxide that has resulted in their association with autotrophs, than demonstrated proof of their ability to develop at the expense of CO_2. . . . The problem which is difficult to resolve is: does the organism incorporate organic C directly in the reduced state, or only reassimilate the CO_2 it has produced during oxidation of the reduced organic carbon. . . .

The definition of autotrophy as the ability of an organism to synthesize all of its cell material (except perhaps traces of vitamin growth factors) from CO_2 is clear and precise. Thus, there is no case at all for describing the type of metabolism encountered in the methane- or methanol-grown organisms as autotrophic and, at this stage, there seems to be no need to introduce a term other than heterotrophic. The introduction of the term "pseudo autotroph" to describe *Pseudomonas* PRL-W4 (70) is, in the reviewer's opinion, unnecessary and liable to lead to confusion. In the field of C_1 metabolism the ratio:

$$\frac{\text{amount of cell material made from reduced } C_1 \text{ units}}{\text{amount of cell material made from } CO_2}$$

may well be found to vary with the reduction level of the substrate, and, as Leadbetter & Foster's experiments indicate (94), may be of the same order

as found in "classical" heterotrophs. The only case in which difficulty arises, and this is only a semantic difficulty, is when a strictly autotrophic type of metabolism is encountered during growth on an organic substrate, e.g., growth on formate by *P. oxalaticus*. This is likely to be a rare type of metabolism; it might even be confined to growth on formate by some, but not all, microorganisms. Such organisms can be classified as either heterotrophic or autotrophic, depending on whether the emphasis is laid on the nature of the growth substrate or the mode of carbon assimilation. Elsden (46) has recently presented a strong case for emphasizing the ability of an organism to synthesize cell material from CO_2, and attaching less importance to the source of the necessary electrons. He suggests the abandonment of the term "chemolithotrophy" and a reinstatement of the older term "chemoautotrophy" (130) as " . . . the ability of an organism to synthesize its cell material from CO_2 using chemical as opposed to radiant energy, the origin of the CO_2 so used being of little significance. . . . " There is much to be said for this assessment, which obviously embraces the property of such organisms as *P. oxalaticus*, and could also include such extraordinary microorganisms as the pseudomonad which may possibly grow in a mineral medium on carbon monoxide (77).

Anaerobic growth on organic C_1 compounds.—Among the microorganisms that are able to ferment organic growth substrates to methane are three which are able to grow on the C_1 compounds, methanol or formate, anaerobically as sole carbon and energy source (see Table II). The bacteria produce from these substrates, cell material, CO_2, methane, and in the case of fermentation of formate, hydrogen as well. Using deuterium-labelled methanol, it has been shown (127, 128, 166) that fermentation of methanol by *Methanosarcina barkeri* may be represented by:

$$4CH_3OD \rightarrow 3CH_3D + CO_2 + HDO + H_2O \qquad \text{(xiii)}$$

the methyl group of methanol being incorporated intact into methane. Less than 1 per cent of the methane was derived from CO_2. Stadtman & Barker carried out experiments (167), using ^{14}C formate, with formate-grown *Methanobacterium formicicum* in order to see whether the methane that was formed, together with CO_2 and hydrogen, during fermentation of formate originated from reduction of formate or of CO_2. An answer could not be obtained, however, owing to the occurrence of a rapid exchange of carbon between formate and CO_2.

The origin of the methane in the fermentation of methanol (or acetate) contrasts with that observed in fermentation of all other substrates tested, where the methane has been shown to be derived almost exclusively from the reduction of CO_2. The evidence for this generalisation has been reviewed by Barker (7). It is unlikely that methanol, formaldehyde, or formate are intermediates in the reduction since the methanol fermenter, *M. barkeri*, is unable to reduce formate or formaldehyde, and the formate fermenter,

M. formicicum, is unable to reduce formaldehyde or methanol (78). Both species can, however, reduce CO_2 with hydrogen.

Barker has presented (7) a metabolic scheme (Fig. 2) which attempts to rationalise the information obtained from fermentation of various substrates. In this scheme an unknown carrier or series of carriers, designated by X, accepts C_1 units arising from CO_2, methanol, or acetate, and reduction reactions enable the C_1 unit to be transformed ultimately into methane.

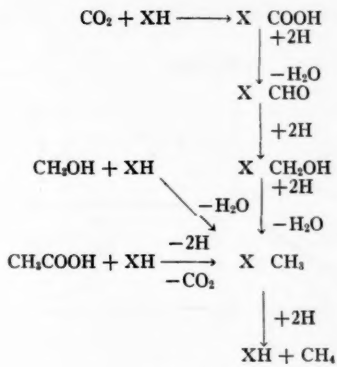


FIG. 2

The interrelation between the fermentation of methanol and acetate has been explored further by Pine & Vishniac (128). They showed that formate is an intermediate in the conversion of methanol to CO_2 and, hence, that the oxidation of methanol is partly, at least, independent of the route by which methane is formed. The crude enrichment culture used was able to adapt from fermentation of acetate to fermentation of methanol, and during the adaptation period the organisms could ferment both substrates simultaneously, the methyl group of both compounds being incorporated intact into methane. It was found that the presence of acetate partly suppressed CO_2 production from methanol, while, in contrast to fermentation of acetate alone, the methyl group of acetate then formed substantial quantities of CO_2. The authors interpreted these results as indicating that acetate dismutation proceeds via methanol, and amended Barker's scheme to that shown in Figure 3. It is suggested that the presence of reducing power, generated by the oxidation of acetate to methanol, caused methanol to be reduced rather than oxidized to CO_2. Pine & Vishniac were not able to detect an exchange between exogenous methanol and the intermediary methanol presumed to be formed from acetate.

Most of the biochemical work done on the methane bacteria has concen-

trated on the mechanism of formation of methane. The routes by which cell constituents are made remain completely unknown, a situation that undoubtedly arises from the great difficulties involved in the isolation and growth of cultures. At this stage, a radioautographic analysis of the entry of labelled substrates into the cellular metabolites, if experimentally practicable with such difficult organisms, might well be the most profitable experimental approach. It could also be most important if the recent finding (165) of adenine-B_{12} coenzyme in *Methanobacterium omelianskii* and *Methanococcus vannielli*, and the observation (116) of the high production of vitamin B_{12} by

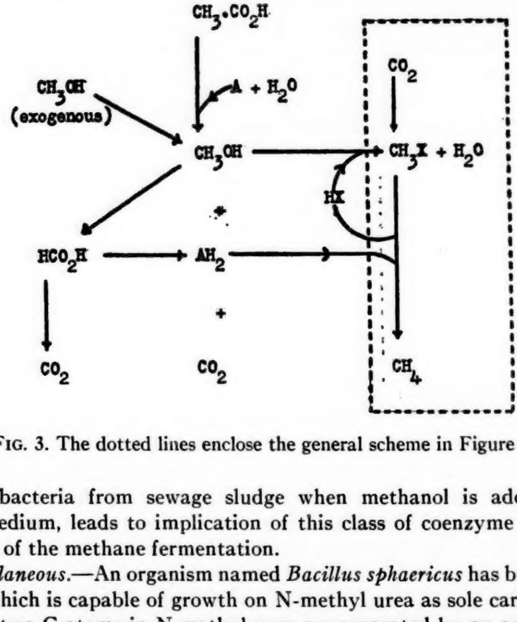


FIG. 3. The dotted lines enclose the general scheme in Figure 2.

methane bacteria from sewage sludge when methanol is added to the growth medium, leads to implication of this class of coenzyme in the biochemistry of the methane fermentation.

Miscellaneous.—An organism named *Bacillus sphaericus* has been isolated (26, 27) which is capable of growth on N-methyl urea as sole carbon source. Since the two C atoms in N-methyl urea are separated by an amino group, the biosynthesis of cell constituents by this organism must, in effect, be carried out from C_1 units. The nature of the C_1 units thus involved is not known. Iyer & Kallio (66) have shown that in this organism the oxidation of methyl urea proceeds by the following steps:

$$CH_3 \cdot NH \cdot CONH_2 + H_2O \rightarrow CH_3NH_2 + CO_2 + NH_3 \text{ anaerobic} \quad \text{(xiv)}$$
$$CH_3NH_2 + \tfrac{1}{2}O_2 \rightarrow HCHO + NH_3 \quad \text{(xv)}$$
$$HCHO + O_2 \rightarrow CO_2 + H_2O \quad \text{(xvi)}$$

The mechanism of the oxidation of formaldehyde is not known and these workers suggest, on the basis of the inability of the cells to oxidize formic acid, that it may not, in fact, proceed via this acid as an intermediate.

Various workers (28, 42, 61, 71) have reported evidence that microbial methane or methanol oxidation proceeds as follows:

$$CH_4 \rightarrow CH_3OH \rightarrow HCHO \rightarrow HCO_2H \rightarrow CO_2 \qquad \text{(xvii)}$$

The enzymes involved at each stage have not been characterized in some organisms, nor have any been purified.

Incorporation of C_1 Units into C_2, C_3, and C_4 Compounds

The discussion so far has been concerned with the primary assimilation processes of microorganisms which grow on C_1 compounds as sole source of carbon. The biosynthetic problems facing such organisms, which have to synthesize the entire carbon content of their protoplasm from C_1 units, are obvious. Nevertheless, their unique problems can be narrowed down essentially to the synthesis of C_4 compounds; once this is accomplished, the biosynthetic reactions leading therefrom to the synthesis of most cell constituents (88) are not expected to be significantly different from heterotrophs growing on carbon sources containing more than three carbon atoms. The crucial problem of biosynthesis of C_4 compounds is not confined to organisms growing on C_1 compounds, but it also faces most heterotrophic organisms to a varying degree. The following section surveys those reactions, whose occurrence is not restricted to microorganisms growing on C_1 compounds as sole carbon source, which may have a role in the synthesis of intermediary metabolites containing two to four carbon atoms.

Incorporation of C_1 units into C_2 compounds.—Evidence has been found with several species of bacteria which points to unknown pathways of formation of C_2 compounds from C_1 units. A clear indication came from the work of Wieringa (204) who isolated an organism, *Clostridium aceticum,* which appeared to carry out a reduction of CO_2 to acetate:

$$2CO_2 + 4H_2 \rightarrow CH_3CO_2H + 2H_2O \qquad \text{(xviii)}$$

In this case, as in the methane fermentation, the CO_2 could be regarded as ultimate oxidant, acetic acid being the final product instead of methane. Several instances have since been reported where a similar reaction system might be operating. One such case is in the fermentation of glucose to acetate by *Clostridium thermoaceticum:*

$$C_6H_{12}O_6 \rightarrow 3CH_3 \cdot CO_2H \qquad \text{(xix)}$$

It was found by Barker & Kamen (9) that in the presence of $^{14}CO_2$ the acetate contained an approximately equal concentration of isotope in the methyl and carboxyl positions. It was therefore suggested that the fermentation occurred as follows:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3 \cdot CO_2H + 8H + 2CO_2 \qquad \text{(xx)}$$
$$8H + 2\,^{14}CO_2 \rightarrow {}^{14}CH_3 \cdot {}^{14}CO_2H \qquad \text{(xxi)}$$

Mass spectrometer studies by Wood (208), using $^{13}CO_2$, showed that indeed approximately 30 per cent of the acetate molecules synthesized by growing

cells in the presence of this tracer were of the species $^{13}CH_3 \cdot {}^{13}CO_2H$. Since the carboxyl group of acetate underwent exchange with CO_2 under the experimental conditions, the evidence for the primary formation of doubly labelled acetate is not absolutely conclusive. However, further experiments designed to check this, indicated that there was, in fact, a double labelling process on which was superimposed some carboxyl exchange. It was further found that ^{14}C formaldehyde entered acetate giving a methyl to carboxyl ratio of 3.58:1. The conversion was, however, slow. Incorporation of ^{14}C from labelled glycine into acetate also was slow and it was concluded that glycine was not an active intermediate in acetate synthesis. The role of formate in the synthesis of acetate was then investigated by Lentz & Wood (97), following their finding that cells of *C. thermoaceticum* catalyzed a rapid interchange between formate and CO_2. In experiments with pools of both CO_2 and formate in which one of these was labelled with ^{14}C, formate was found to be a better precursor of the methyl group of acetate than was CO^2, while the carboxyl group appeared to be more directly derived from CO_2.

Similar processes may well operate during the fermentation of glucose or lactate mainly to acetate, butyrate, and CO_2 by *Butyribacterium rettgeri*, investigated by Pine & Barker (126). These authors found that fermentation of glucose in the presence of either ^{14}C formate or $^{14}CO_2$ gave rise to labelled acetate in which the labelling patterns were very similar in the case of both tracers. The acetate was labelled with a methyl to carboxyl ratio of 1:1.24. The ^{14}C formate was rapidly converted to $^{14}CO_2$ under the conditions of the experiment, and a differentiation of the two tracers as precursors of the individual carbon atoms of the acetate was not made as in the case of *C. thermoaceticum*. Formaldehyde was metabolized to a limited extent with preferential incorporation into the methyl group of acetate.

The reactions involved in producing this striking labelling pattern in acetate still remain unknown. Carbon 1 fixation by glycine to form serine, which is transformed into pyruvate and then acetate, forms the basis of one theory to account for the labelling of the methyl carbon of acetate (192). This is rendered unlikely by the experiment of Pine & Barker (126) in which *B. rettgeri* was incubated with $^{14}CO_2$ for 5 min. and the radioactive products formed in the cells separated and identified by chromatography and radioautography. The most prominent radioactive products were acetate and lactate, while glycine and serine did not contain a detectable amount of ^{14}C. Non-labelling of glycine and serine from $^{14}CO_2$ by this organism was also observed by Lynch & Calvin (100).

Barker, Ruben & Beck showed in 1940 (10) that the fermentation of uric acid to acetic acid, ammonia, and CO_2 by *Clostridium acidi-urici*, in the presence of $^{11}CO_2$, resulted in the acetate being doubly labelled. With the example of *C. aceticum* in mind, the authors suggested that the fermentation might involve a complete oxidation of the purine coupled with a reduction of CO_2 to acetic acid. This view was modified when later studies, using ^{14}C substrates (8, 73), showed that only about one-fifth of the acetate was derived from CO_2, the remaining four-fifths originating from the carbon of

the uric acid. The main pathway of fermentation was shown, by data from several groups of workers [see (151)], to follow the sequence:

$$\text{Purine} \rightarrow \text{formate} + \text{glycine} \rightarrow \text{serine} \rightarrow \text{pyruvate} \rightarrow \text{acetate} + CO_2 \qquad \text{(xxii)}$$

Since CO_2 was shown to exchange rapidly with formate, the appearance from $^{14}CO_2$ of isotope in the methyl group of acetate was readily explicable. On the other hand, the appearance of $^{14}CO_2$ in the carboxyl group of acetate is puzzling, and indicates that scheme (xxii) may not express the fermentation process completely.

Evidence for the incorporation of C_1 units into glycine, or a closely related compound, by methanol- or formate-grown *Pseudomonas* AM1 has been presented earlier in this review. This may be related to the rapid incorporation of $^{14}CO_2$ into the carboxyl group of both intra- and extracellular glycine by *Brucella abortus* (104, 181). Forty per cent of the total $^{14}CO_2$ incorporation by this organism, when grown in a complex medium, was found in the carboxyl group of glycine. Although derivation of the carboxyl group of glycine from CO_2 has been found with other microorganisms (8, 36, 38, 44, 182, 184), the relative extent of this $^{14}CO_2$ incorporation appears to be of a higher order of magnitude in the case of *B. abortus*. The fact that $^{14}CO_2$ was incorporated into the carboxyl group of extracellular glycine by "resting" cells, while ^{15}N from $(^{15}NH_4)_2SO_4$ was not, shows that the—CH_2NH_2 grouping of the glycine is formed before the carboxyl group is formed from, or exchanged with, the $^{14}CO_2$.

Incorporation of C_1 units into C_3 compounds.—The possibility that a direct C_1+C_2 condensation may occur in microbial synthesis of cell constituents has exercised the minds of biochemists for many years. The clearest evidence that has pointed to this possibility has come from the study of labelling patterns in amino acids obtained from *C. kluyveri* grown on media containing $^{14}CO_2$ or 1-^{14}C acetate (182, 183, 184), and from *R. rubrum* grown photosynthetically in the presence of $^{13}CH_3 \cdot {}^{14}CO_2H$ (37, 38). As mentioned previously, these studies showed that the carboxyl group of alanine was derived almost exclusively from CO_2, and the α- and β-carbon atoms from the carboxyl and methyl groups of acetate, respectively, suggesting the formation of pyruvate by the addition of CO_2 to an acetyl unit. Stanier and his coworkers have also suspected that CO_2 may play some role in the formation of C_3 compounds from acetate by *R. rubrum* (168). Despite these data, no evidence has yet been found in cell-free extracts for a C_2+C_1 condensation providing a satisfactory explanation for the labelling data in these two instances, although it may be speculated whether the CO_2-pyruvate exchange system recently found in *C. kluyveri* (157) might be involved. This reaction will be referred to again later.

There are four distinct types of C_2+C_1 condensation, listed below, which are known to lead to the net synthesis of C_3 compounds. The full role of some of these in cell constituent biosynthesis has yet to be evaluated. (*a*) The biotin-dependent carboxylation of acetyl CoA, by a C_1 unit donated from the β-carboxyl of oxalacetate, to give malonyl CoA. (*b*) The direct carboxyla-

tion of acetyl CoA to give malonyl CoA, catalyzed by a biotin-containing enzyme:

$$\text{acetyl CoA} + CO_2 + \text{ATP} \xrightarrow{Mn^{++}} \text{malonyl CoA} + \text{ADP} + P_i \qquad \text{(xxiii)}$$

(*c*) The tetrahydrofolate-dependent formylation of glycine to give serine, catalyzed by the enzyme serine hydroxymethylase:

$$\text{“}CH_2OH\text{”THF} + CH_2NH_2\cdot CO_2H \rightarrow CH_2OH\cdot CHNH_2\cdot CO_2H + \text{THF} \qquad \text{(xxiv)}$$

(*d*) The reversal of pyruvate decarboxylation.

Reaction (*a*), a representative of a new class of reactions termed transcarboxylations, has been found in extracts of *Propionibacterium shermanii* (180) and is dealt with more fully later in the review.

Reaction (*b*), first found in extracts of avian liver (198) and pig heart and pigeon liver (49), is not confined to animal tissue as similar reactions have been observed in cell-free yeast extracts (101) and in extracts of *Mycobacterium avium*, *Mycobacterium smegmatis*, and *Nocardia asteroides* (89). The participation of biotin in these reactions is of great interest, in view of its hitherto unknown role in carboxylation reactions. Lynen and co-workers (102) have studied the mechanism of the biotin involvement in a similar reaction, the carboxylation of β-methyl crotonyl CoA to give β-methyl glutaconyl CoA in extracts of *Mycobacterium:*

$$CH_3\text{—}\underset{}{\overset{\displaystyle CH_3\atop|}{C}}\text{=}CH\cdot CO\cdot CoA + CO_2 + ATP$$

$$\rightarrow HOOC\cdot CH_2\text{—}\overset{\displaystyle CH_3\atop|}{C}\text{=}CH\cdot CO\cdot CoA + ADP + P_i \qquad \text{(xxv)}$$

It is not yet known whether the enzyme, biotin, CO_2, and ATP react together in a concerted fashion, or whether intermediate complexes of the reactants exist.

The further extension of these studies to bacterial systems will be of considerable interest, although it is difficult as yet to foresee that carboxylation of acetyl CoA to malonyl CoA could fulfill the role of the postulated C_2+C_1 condensation in *C. kluyveri* and *R. rubrum*. Its main impact so far has been in the field of lipid synthesis [for review, see Stumpf (174)].

Reaction (*c*) is one of the most extensively investigated of all of the C_1+C_2 condensations and has been reviewed recently in detail elsewhere (63, 144). The topic will not be duplicated in this review except to draw attention to those instances where the system may form an integral part of the main carbon incorporation pathway for microorganisms growing on C_1 and C_2 compounds as sole carbon sources. One such organism is *Diplococcus glycinophilus* which grows anaerobically by fermenting glycine to acetate and CO_2 (30):

$$4CH_2NH_2\cdot CO_2H + 2H_2O \rightarrow 3CH_3\cdot CO_2H + 2CO_2 + 4NH_3 \qquad \text{(xxvi)}$$

Gunsalus and co-workers (58) have proposed that the fermentation proceeds by the following series of reactions:

$$\text{Glycine} \rightarrow \text{“}C_1\text{”THF} + CO_2 + NH_3 \quad \text{(xxvii)}$$
$$\text{Glycine} + \text{“}C_1\text{”THF} \rightarrow \text{serine} + \text{THF} \quad \text{(xxviii)}$$
$$\text{Serine} \rightarrow \text{pyruvate} + NH_3 \quad \text{(xxix)}$$
$$\text{Pyruvate} \rightarrow \text{acetate} + CO_2 \quad \text{(xxx)}$$

The coupled breakdown of pyruvate could provide the energy necessary for the synthesis of cell constituents, the first step being the synthesis of serine as in reaction (xxviii). Experiments by Geller (53), with a coryneform bacterium grown on glycine as sole source of both carbon and nitrogen, have indicated a similar pathway of biosynthesis, the energy in this case being derived by aerobic oxidation of glycine. Evidence for the operation of serine hydroxymethylase on the main pathway of carbon incorporation from methanol by *Pseudomonas* PRL-W4 and *H. vulgare*, and from methanol and formate by *Pseudomonas* AM1, has already been presented.

The study of reaction (*d*) goes back to the discovery in 1944 (98, 194, 195) that the breakdown of pyruvate by *E. coli* to acetyl phosphate and formate, the "phosphoroclastic reaction," was reversible:

$$CH_3\cdot CO\cdot COOH + H_3PO_4 \rightleftharpoons CH_3\cdot COOPO_3H_2 + HCOOH \quad \text{(xxxi)}$$

Subsequent work on this system and similar ones in other organisms has shown the reaction to be deceptively simple in appearance.

The cofactor requirements for the oxidation of pyruvate to acetyl CoA, DPNH, and CO_2 by *E. coli* extracts are TPP, CoA, lipoic acid, and DPN. An exchange of $^{14}CO_2$ with the carboxyl group of pyruvate is catalyzed by a protein fraction in the presence of TPP (172), indicating the reversible reaction:

$$CH_3\cdot CO\cdot CO_2H \xrightleftharpoons{\text{TPP}} [CH_3CHO\cdot TPP] + CO_2 \quad \text{(xxxii)}$$

The mechanism of the exchange of formate with the carboxyl group of pyruvate is not fully understood. The required cofactors are CoA, TPP phosphate, and Mg^{++} (31, 173) and exchange is also stimulated by ATP and tetrahydrofolate (32).

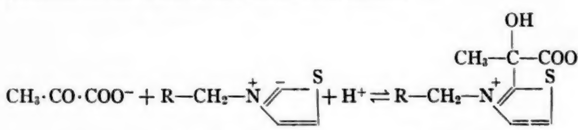
Another extensively investigated system is that present in *Clostridium butyricum*, which degrades pyruvate to acetyl phosphate, CO_2, and hydrogen. The cofactors for the degradation are CoA, TPP, phosphate, and Fe^{++}; lipoic acid is apparently not involved (206). Formate is not present as an end product, nor does it exchange with pyruvate, although CO_2 readily does so (205). The cofactors for the CO_2 exchange are CoA, TPP, phosphate, or arsenate (113, 207) and derivatives of vitamin B_{12} (145). It is suggested that the C_2 compound active in the exchange process is at the aldehyde level of oxidation. The enzyme system has been partially fractionated by ethanol precipitation (113) but is found to be extremely labile. The reaction has been reversed with crude extract, acetyl phosphate, CO_2, and sodium hydrosul-

phite, giving rise to very small quantities of pyruvate, amounting to 0.4 per cent of the acetyl phosphate added (112).

The complexity of this field is emphasized by studies on *Bacillus macerans* (60), an organism which ferments carbohydrates to ethanol, acetone, acetic and formic acids, hydrogen, and CO_2. This organism appears to exhibit a combination of the pyruvate breakdown system of *E. coli* and *C. butyricum*. Whole cells catalyze both $^{14}CO_2$ and ^{14}C formate exchange with pyruvate. In cell-free extracts the former activity is completely recovered but the latter is lost, showing that formate and CO_2 are exchanging via pathways which must be different; to what extent is not yet known.

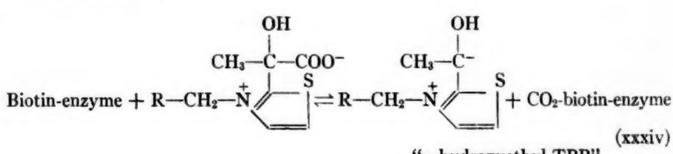
Similarly complex results have been obtained with *Micrococcus lactilyticus*, extracts of which, at pH 6.5, oxidatively decarboxylate pyruvate to acetate, CO_2, and hydrogen (202); at pH 8.5, however, the products are acetate and formate (105). Formate is not decomposed to CO_2 and hydrogen at either pH, and at an alkaline pH formate is exchanged into the carboxyl of pyruvate (105, 120). The enzymes participating in this exchange have been separated from those catalyzing oxidative decarboxylation by ammonium sulphate fractionation (106, 107). Dialysis of the exchange enzyme system resulted in loss of formate-exchanging activity, which could be restored with boiled cell extract. Identification of the cofactor(s) responsible is awaited with interest. A search is also being made for the factor(s) present in yeast extract which give(s) rise to high levels of pyruvate-formate exchange activity in *Streptococcus faecalis* 10Cl (209). The evidence indicates an involvement of tetrahydrofolic acid.

Shuster & Lynen have recently implicated biotin as a cofactor in a CO_2-pyruvate exchange system operating in *C. kluyveri* (157). The exchange reaction is completely inhibited by avidin, no such inhibition being observed on pretreatment with biotin. Other cofactors are TPP, ATP, and magnesium ions. In contrast with some other CO_2-pyruvate exchange systems, neither a CoA nor an orthophosphate dependence could be shown. The authors have postulated the following reaction sequence:



(xxxiii)

Thiazole ring of TPP in form of thiazolium zwitterion — "α-TPP-lactic acid"



(xxxiv)

"α-hydroxyethyl-TPP"

$$\text{CO}_2\text{-biotin-enzyme} + \text{ADP} + \text{P}_i \rightleftharpoons \text{Biotin-enzyme} + \text{CO}_2 + \text{ATP} \qquad \text{(xxxv)}$$

$$\text{Sum: Pyruvate} + \text{TPP} + \text{ADP} + \text{P}_i \rightleftharpoons \text{“}\alpha\text{-hydroxyethyl-TPP”} + \text{CO}_2 + \text{ATP} \qquad \text{(xxxvi)}$$

The thiamin compounds in reactions (xxxiii) and (xxxiv) have previously been proposed as intermediates in thiamin-catalyzed decarboxylations [see (109)]. Shuster & Lynen point out the similarity in electronic structure of "α-TPP-lactic acid" to a β-ketoacid, rendering the carboxyl group susceptible to nucleophilic attack by biotin. The extent of biotin involvement in other CO_2-pyruvate exchange systems remains to be determined.

Other investigations of the interrelation between pyruvate and C_1 compounds have been carried out in *C. acidi urici* (150), a rumen organism LC (125), and *Zymosarcina ventriculis* (19, 20, 21).

In spite of the rapidity of the formate (or CO_2)-pyruvate exchange reactions in cell-free extracts, the net synthesis of pyruvate by a reversal of such reactions has been demonstrated to only a very limited extent, and the evidence is, as yet, against any major role for the reaction in synthesis of cell constituents.

Miscellaneous.—The enzyme glyoxylic acid carboligase catalyzes the condensation and decarboxylation of two molecules of glyoxylate to one of tartronic acid semialdehyde (86, 87):

$$2\text{CHO}\cdot\text{CO}_2\text{H} \xrightarrow{\text{TPP}} \text{CHO}\cdot\text{CHOH}\cdot\text{CO}_2\text{H} + \text{CO}_2 \qquad \text{(xxxvii)}$$

This has been shown to be a key reaction in the microbial synthesis of cell constituents from glycollate (81, 82) and oxalate (136, 141, 142). If the reaction involves an initial decarboxylation of one molecule of glyoxylate to form a C_1-fragment at the level of formaldehyde, which then reacts by an aldol condensation with a further molecule of glyoxylate to give the product, then this reaction may be regarded as a C_2+C_1 type of condensation.

Dickens & Williamson have shown (41) that transketolase (obtained from bakers' yeast) catalyzes a transfer of a glycolyl fragment from hydroxypyruvate to formaldehyde:

$$\text{CH}_2\text{OH}\cdot\text{CO}\cdot\text{COOH} + \text{HCHO} \rightarrow \text{CH}_2\text{OH}\cdot\text{CO}\cdot\text{CH}_2\text{OH} + \text{CO}_2 \qquad \text{(xxxviii)}$$

This reaction may be classified as a kind of C_2+C_1 condensation even though it results only in the reduction of one C_3 compound to another.

Incorporation of C_1 units into C_4 compounds.—In practically all cases discovered so far, condensations of this type involve CO_2 as the C_1 unit. The formation of carbon to carbon bonds by CO_2 fixation is normally an endergonic reaction which requires an external supply of energy. This energy is usually supplied through coupled oxidoreduction reactions of pyridine nucleotide coenzymes, or the participation of "energy-rich" phosphate compounds. The mechanisms of three distinct C_3+CO_2 carboxylation reac-

tions of this type have been extensively studied in the past fifteen years [for review see (197)].

$$\text{pyruvate} + CO_2 + \text{TPNH} + H^+ \overset{Mn^{++}}{\rightleftharpoons} \text{malate} + \text{TPN}^+ \quad \text{(xxxix)}$$
$$\text{phosphopyruvate} + CO_2 + \text{IDP (or GDP)} \rightleftharpoons \text{oxaloacetate} + \text{ITP (or GTP)} \quad \text{(xl)}$$
$$\text{phosphopyruvate} + CO_2 \rightarrow \text{oxaloacetate} + \text{Pi} \quad \text{(xli)}$$

Reaction (xxxix) catalyzed by the well-known "malic enzyme" is widely distributed in bacteria (121, 197). Reaction (xl) was discovered in chicken liver preparations by Utter and his co-workers (190, 191, 193), and evidence for its existence in many microorganisms has since been obtained, e.g., in autotrophically-grown *Hydrogenomonas* (68); *P. shermanii* (129); *P. oxalaticus* (134); *T. thiooxidans* (178); bakers' yeast, an ADP-specific enzyme (29); *Aspergillus niger*, an ADP-specific enzyme (210); and *Nocardia corallina* (22). The irreversible carboxylation of phosphopyruvate, reaction (xli), discovered in spinach leaves by Bandurski & Greiner (6), has also been found in bacteria, e.g., *T. thiooxidans* (176, 178), and *P. shermanii* (129).

Important new discoveries in the field of carboxylation reactions have centred around the carboxylation of propionate to succinate and the reverse reaction. This work has directly implicated both biotin and the B_{12} coenzymes recently discovered by Barker and his colleagues (11, 12, 13, 200). Propionic acid is formed by the propionibacteria from fermentation of glucose, lactose, or in some cases from pentoses, and it has generally been considered that these carbohydrates are catabolized to pyruvate which is then converted to oxalacetate by CO_2 fixation. Oxalacetate is then reduced to succinate, which is converted to succinyl CoA and decarboxylated to propionyl CoA and CO_2. Because succinate is symmetrical, the CO_2 evolved would come in equal parts from the original fixation and from the carboxyl group of pyruvate. However, the results of experiments reported by several workers in the last few years indicated that a C_1 unit other than free CO_2 was a product of the cleavage. This work has been reviewed by Swick & Wood (180). The discovery (48) that carboxylation of propionate to succinate in animal tissue went via the sequence:

$$CH_3 \cdot CH_2 \cdot CO_2H \xrightarrow{\text{CoA}} CH_3 \cdot CH_2 \cdot CO \cdot \text{CoA} \xrightarrow{CO_2} \underset{\text{Methyl malonyl CoA}}{CH_3 \cdot CH(CO_2H) \cdot CO \cdot \text{CoA}}$$
$$\longrightarrow CO_2H \cdot CH_2 \cdot CH_2 \cdot CO \cdot \text{CoA} \quad \text{(xlii)}$$

led Swick & Wood (180) to investigate whether the reversal of such a scheme operated during the propionic fermentation. Their results obtained with *P. shermanii* showed this to be the case, with one important exception, viz., the decarboxylation of methyl malonyl CoA was accomplished by the enzymic transfer of the carboxyl group to an acceptor molecule, pyruvate:

$$\text{Succinyl CoA} \rightleftharpoons \text{methyl malonyl CoA} \quad \text{(xliii)}$$
$$E + \text{methyl malonyl CoA} \rightleftharpoons \text{propionyl CoA} + E - \text{"}C_1\text{"} \quad \text{(xliv)}$$
$$E - \text{"}C_1\text{"} + \text{pyruvate} \rightleftharpoons \text{oxalacetate} + E \quad \text{(xlv)}$$
$$\text{oxalacetate} + 4H \rightleftharpoons \text{succinate} \quad \text{(xlvi)}$$
$$\text{succinate} + \text{propionyl CoA} \rightleftharpoons \text{succinyl CoA} + \text{propionate} \quad \text{(xlvii)}$$

$$\text{Sum: Pyruvate} + 4H \rightleftharpoons \text{propionate} \quad \text{(xlviii)}$$

Reactions (xliv) and (xlv) involve a new biochemical reaction, termed a transcarboxylation, and provide an explanation for the non-involvement of free CO_2 in the decarboxylation of succinate. Biotin was found to be a cofactor for the transcarboxylation, indicating a role similar to that found in carboxylation of acetyl CoA (49, 101, 198), β-methyl crotonyl CoA (102), and the direct carboxylation of propionyl CoA (74). Some experiments were performed with *P. shermanii* extracts to test other compounds as donors and acceptors in transcarboxylation. It was found that oxalacetate and acetyl CoA gave rise to malonyl CoA, while oxalacetate and butyryl CoA gave rise to ethyl malonyl CoA; malonyl CoA and ethyl malonyl CoA transcarboxylated with propionyl CoA yielding, presumably, methyl malonyl CoA. Swick & Wood concluded that the apparent structural requirements for a donor compound are that it has a carbonyl group β to the carboxyl group being donated and adjacent to another carboxyl group as in oxalacetate, or to coenzyme A, as in methyl malonyl CoA. Almost simultaneously with this work, Lynen and his colleagues reported experiments (164) with *P. shermanii* leading to essentially the same results. In addition, these workers were able to show that the isomerization reaction was dependent on the presence of dimethylbenziminazole-B_{12} coenzyme. Similar findings have been made with animal tissue preparations (59, 96, 162, 164). In view of the role of this coenzyme in isomerization of glutamate to β-methyl aspartate by *Clostridium tetanomorphum* (11), and of succinyl CoA to methyl malonyl CoA, it has been termed a coenzyme of transpropionation by Stern & Friedman (169). Eggerer *et al.* have shown that in the latter rearrangement the thiolester group migrates and not the free carboxyl group, and have proposed an intramolecular mechanism for the rearrangement (43).

Carboxylation of propionate to succinate has been observed with *C. limicola* (91) and *R. rubrum* (33, 34, 35). The reaction system in *R. rubrum* has been shown (55) to be similar to that described above in animal tissue and *P. shermanii.*

The old question of carboxylation of pyruvate itself to form oxalacetate ("Wood-Werkman reaction") has been reopened by Utter & Keech (189). These authors have partially purified a system from avian and beef liver which catalyzes the following reaction:

$$\text{Pyruvate} + \text{ATP} + CO_2 \underset{Mg^{++}}{\overset{\text{acetyl CoA}}{\rightleftharpoons}} \text{oxalacetate} + \text{ADP} + P_i \qquad \text{(xlix)}$$

The reaction is absolutely dependent on catalytic amounts of acetyl CoA and is inhibited by avidin, an effect which is nullified by pretreatment with biotin. The enzyme preparation is free of phosphopyruvate carboxykinase. When 1-^{14}C acetyl CoA is present in the reaction mixture it does not enter oxalacetate. The mechanism of the reaction has not yet been elucidated; the attractive possibility of a transcarboxylation mechanism based on an initial carboxylation of acetyl CoA to give malonyl CoA, followed by carboxyl transfer to the methyl group of pyruvate, has not received experimental support. It will be interesting to see if such a system occurs in microorgan-

isms, e.g., in the systems responsible for CO_2 fixation into oxalacetate in *E. coli* and *Proteus morganii* (69), and *A. niger* (210); evidence has been obtained that it may be present in *Chromatium* (52).

Evidence has been presented (158 to 161) that *Rhodopseudomonas gelatinosa* is capable of carboxylating acetone to acetoacetate, both anaerobically in the light, and aerobically in the dark. The mechanism of this carboxylation reaction is not known.

Quantitative Role of CO_2 Fixation in Heterotrophic Growth

Following the survey of C_1-fixation reactions, many of which are carboxylation reactions, it is of interest to examine briefly what is actually known in quantitative terms of the contribution of CO_2 to non-autotrophic bacterial synthesis of cell material. It has been known for a long time that the presence of CO_2 is essential for the growth of many types of microorganisms, and the concept has arisen that one of the essential roles played by CO_2 is in the synthesis of the tricarboxylic acid cycle intermediates, malate and oxalacetate, from pyruvate or phosphopyruvate [for review, see Wiame & Bourgeois (203)]. The synthetic function of the tricarboxylic acid cycle in providing the aspartate and glutamate for protein synthesis would then result in CO_2 being incorporated into these two amino acids and the amino acid families of which they are precursors (88). This was indeed found when Abelson, Bolton & Aldous (1) grew *E. coli* on glucose in the presence of $^{14}CO_2$ for one generation, and analysed the resulting distribution of tracer in the cells. They observed that for each mole of glucose metabolized, 0.21 mole of $^{14}CO_2$ was incorporated, and since only half the glucose carbon was assimilated, this showed that approximately 7 per cent of the cellular carbon had passed through the stage of CO_2 exchangeable with exogenous $^{14}CO_2$. The tracer was distributed between the lipid, nucleic acid, and protein hydrolysate fractions of the cells in the percentage ratio 5:36:58, respectively. The amino acids derived from aspartate and glutamate were found (148) to account for 50 per cent of the total protein carbon, and thus the high incorporation of CO_2 into protein is consistent with the idea of replenishment of the tricarboxylic acid cycle by CO_2 fixation. However, the actual demonstration that CO_2 has been fixed in a net fashion is fraught with the greatest difficulties. This is due not only to overall production of CO_2 by aerobic organisms but also to the occurrence of exchange reactions. Since most of the C_3+CO_2 carboxylations are readily reversible, $^{14}CO_2$ incorporation will always be observed even when no net replenishment of the cycle is necessary, e.g., when C_4 or C_5 acids are growth substrates. Both exchange processes into, and net replenishment of, C_4 acids by C_3+CO_2 fixations can result in incorporation of up to 25 per cent of carbon from CO_2 into tricarboxylic acid cycle intermediates. When the C_4 acid has been transformed to α-ketoglutarate prior to amination to glutamic acid, this maximum figure would drop because of the addition of an acetate unit by citrate condensing enzyme, and loss of labelled CO_2 by decarboxylation of oxalsuccinate. Since

50 per cent of the protein in *E. coli* is derived from the aspartic and glutamic families, up to 12 per cent of the protein carbon might be expected to be exchangeable with $^{14}CO_2$, or rather less for total cell carbon (203).

Figures of this order have been obtained by several investigators. Leadbetter & Foster (94) estimated that 21 to 26 per cent and 28 to 33 per cent of the total cell carbon of glucose-grown *E. coli* and *P. fluorescens*, respectively, was exchangeable with CO_2, although, as the authors point out, these figures are probably too high. Tomlinson & Barker (184) obtained the figure of 15 per cent of total cell carbon being exchangeable with $^{14}CO_2$ for *C. kluyveri* grown on acetate, ethanol, and bicarbonate; this figure was raised to 28 per cent if the yeast extract in the medium was replaced by biotin and *p*-aminobenzoic acid. In the case of this organism an actual net CO_2 fixation does take place. Wiame & Bourgeois (203) investigated the amount of $^{14}CO_2$ incorporation into *Bacillus subtilis* as a function of the growth substrate. They found that the substrates could be divided broadly into two classes, giving respectively high and low figures for incorporation. Those giving high figures were glucose, arabinose, and glycerol, while citrate and malate gave low figures. The authors suggest that the higher CO_2 incorporation observed with substrates giving rise to C_3 compounds is the result of replenishment of the tricarboxylic acid cycle by CO_2 fixation, although pyruvate occupied the anomalous position of lying between these two groups. This may indeed be so, although the profound differences in enzyme content attendant on change of growth substrate may also considerably affect the rate of exchange processes. The magnitude of the changes in enzyme levels accompanying change of growth substrate is illustrated by data of Kornberg (80) on *Pseudomonas ovalis* Chester: the specific activities of citrate-condensing enzyme in cells grown on citrate, aspartate, and acetate were found to be 2.1, 5.4, and 13 units, respectively. Such data raise questions as to the differences in the levels of C_3+CO_2 carboxylation enzymes that may also occur with change of substrate, and the effect of such enzymic differences on the extent of exchange processess. Factors such as these may well mask the difference in net $^{14}CO_2$ fixation that may be expected, for example, between growth on C_3 and C_4 substrates.

Measurements of the amount of cellular carbon that is exchangeable with $^{14}CO_2$ do have great value in deciding whether carboxylation reactions of a large order of magnitude are operating on the main pathway of carbon assimilation. Kornberg & Quayle (83) measured the drop in specific activity of cellular protein when cultures of *Pseudomonas* KB1 growing on uniformly labelled acetate were flushed with air-CO_2 (95:5, v/v). The specific activity dropped by 15 to 17 per cent. If it is assumed that equilibration between intra- and extracellular CO_2 is reasonably complete, this finding ruled out, as being of major importance, the occurrence of C_2+CO_2 reactions, where at least 33 per cent of the cellular carbon would be expected to be exchangeable with CO_2. The assumption of a high level of CO_2 equilibration, under these steady-state experimental conditions, seems reasonable in view of the

finding that similar CO_2 flushing reduced the specific activity of non-volatile cellular carbon of formate-grown *P. oxalaticus* by 93 per cent. The 15 to 17 per cent drop in specific activity of protein observed in acetate-grown *Pseudomonas* KB1, lends strong support for caution in taking $^{14}CO_2$ exchange to be an indicator of net CO_2 fixation into the tricarboxylic acid cycle. Acetate-grown *Pseudomonas* KB1 need no mechanism for net replenishment of tricarboxylic acid cycle intermediates by CO_2 fixation, as the glyoxylate cycle shown to operate in this organism performs this very function (79). Despite this fact, more $^{14}CO_2$ is found in the protein than is predicted (12 per cent) on the basis of synthesis of all malate and oxalacetate by carboxylation reactions of the type C_3+CO_2.

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PHAGE-HOST RELATIONSHIPS IN SOME GENERA OF MEDICAL SIGNIFICANCE[1]

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Introduction

The motivations for this review are quite diverse. One of the most important is the reviewer's conviction that the comparative approach should be encouraged in thinking and writing about bacteriophage. The decision of phage workers in the 1940's and 1950's to restrict their efforts largely to the coliphages has led to rapid, cumulative advances in understanding phage and its implications for biology. Meanwhile, because it was unorganized, much useful information on non-enteric phages has been virtually lost or ignored. Used consistently, this information would permit verification or modification of existing generalizations, and as the comparative aspects were stressed, would foster the emergence of new inter-relationships. As a prerequisite for these developments, basic information for phage-host systems embracing the taxonomic breadth of bacteriology must be obtained, organized, and assessed. One purpose of this review is to organize and assess information already available for some groups of non-enteric phages.

The choice of phage groups to be reviewed was determined by the reviewer's own interest in phages associated with bacteria of medical importance. Investigation of phages for mycobacteria, streptococci, staphylococci and other bacteria has accelerated in recent years as a result of two developments. The first, growing recognition of the role of bacteriophage in bacterial variation; the second, full appreciation of the potentialities of phage typing in epidemiological investigations. Present interests rest on firmer theoretical grounds than did phage therapy of the preceding era. Phage therapy is no longer seriously considered in medicine, but the role of phage in the genesis of bacterial disease is important and is now being explored. When the functional role of phage as a typing agent is added to this development, it is apparent that the study of these agents has taken on a new perspective in relation to medical problems.

An effort has been made to write this review for the general reader interested in phage and its relation to medical problems, as well as for the phage worker. The reader's familiarity with both the lytic and lysogenic components of the phage life cycle and with common terminology is assumed. Graphic descriptions of the life cycle are so common as to require no specific reference. Orientation to any phase of the cycle can be obtained from Adams' book *Bacteriophages* (1). The reader should consult the glossary of this book for definitions.

[1] The survey of the literature pertaining to this review was concluded in December, 1960.

The presentation of each group of phages is somewhat varied and has grown out of the nature of the material. Although no effort has been made to include every reference, those given should lead the interested reader to the pertinent literature. A section on the corynebacterial phages was planned, but reviews of old and new literature have appeared, making this need less pressing (2 to 4). It is hoped that in the future individuals working with specific phage groups will undertake summaries of their own fields.

Mycobacterial Phages

In 1935, Steenken (5) reported that a culture of virulent *Mycobacterium tuberculosis*, strain H37Rv, began to liquify after three to four months of incubation. In some cases, colonies actually disappeared and clones of avirulent, lysis-resistant organisms appeared. Steenken later found (6) that filtrates of lysed cultures would lyse virulent strains of *M. tuberculosis* suspended in media at pH 4.2 to 5.8, but transmissibility of the lytic factor was not tested. The suspicion that bacteriophage activity was involved has lingered in the literature. The first mycobacterial phage (mycophage) was isolated by Gardner & Weiser in 1947 (7), and two other reports of successful isolations followed (8, 9).

Sources and methods of isolation.—Soil is a consistent source of mycophages. Gardner & Weiser (7) enriched compost and soil samples twice weekly with heavy suspensions of *M. smegmatis* and incubated the mixtures at 37°C. To test for phage, dilutions of the samples were periodically plated on agar along with the enriching strain. Isolations were successful from 3 of 10 enriched samples and from none of 10 unenriched samples. Hauduroy & Rosset (8) simply incubated garden soil in broth and isolated mycophage after adding a filtrate of this material to broth cultures of *M. paratuberculosis*. Penso & Ortali (9), using soil from the vicinity of a manure pile, spotted an aqueous extract of this material on mycobacteria isolated from the same soil, and isolated five phages which could be distinguished by host range, morphology, and antigenicity. Subsequent isolations of mycophages by other workers (11 to 16) were based on the method devised by Gardner & Weiser. In general, enrichment is superior to non-enrichment and Penso & Ortali's success in the absence of enrichment (9) was probably attributable to an indigenous mycobacterial population. The need for continuous enrichment suggests that the added mycobacteria fail to reproduce.

What is the origin of phages isolated by enrichment? They may arise from resident soil mycobacteria but, with one exception (9), no attempt was made to isolate resident strains. Conceivably, highly virulent phage mutants arise in the enriching strain and are thus able to overcome the immunity normally enjoyed by a strain to the phages it carries. Indeed, from descriptions of their plaques, most of these phages appear to be virulent. Support for an endogenous origin comes from observations of Takeya *et al.* (16) that different enriching strains yielded different phages in the same soil while serologically identical phages were isolated from four different soils enriched with one strain.

When a search for phage is instituted in a new group of organisms, the bacteria themselves are usually the most convenient source of phage. The assumption, amply justified by experience, is that many strains are naturally lysogenic. Heroic efforts have been made to demonstrate lysogeny among the mycobacteria (12, 17, 19) but only three phages have been discovered in this manner (18 to 20). Possible reasons for this lack of success will be discussed in the section on lysogeny.

A few attempts have been made to isolate mycophages from other source material. Hnatko (12) screened 42 positive sputa and 17 stool specimens from tuberculosis patients without success. Sputum filtrates were tested on 45 saprophytic and 50 pathogenic strains and passaged with 15 saprophytic and 10 pathogenic strains to no avail. On the other hand, Piguet (21), having observed acid-fast bacilli in the stomach of the larvae of the mosquito *Culex pipiens*, isolated two phages found to be active on both saprophytic and pathogenic mycobacteria from the mosquito stomach.

The host range of mycophages.—Saprophytic mycobacteria were employed in most successful enrichments (7, 9, 10 to 14, 16). While Hnatko (12) reported three successful isolations following enrichment with bovine and human pathogenic strains of *M. tuberculosis*, most attempts to employ either virulent or avirulent human, avian, or bovine tubercle bacilli have been unsuccessful (10, 13, 14). The only successful isolations from lysogenic mycobacteria involved saprophytes (12, 18 to 20) though similar efforts were made with all types of tubercle bacilli (12, 17, 22).

The interest evinced in the host range of mycophages derives from two potential applications. The first is the possibility of distinguishing one group of mycobacteria from another as, for example, the saprophytes from potential disease producers, or virulent from avirulent strains of tubercle bacilli. The second is the development of a phage typing scheme. It is too early to say whether either of these objectives will be accomplished, but certain interesting facts are already at hand.

Host range tests have revealed that some of the phages have species-specific patterns (10, 13, 23) and some have broader activity (9, 14, 16, 24). Hnatko (12) found that enrichment with pathogenic human and bovine strains produced phages active only on saprophytic strains. Froman *et al.* (14) and Takeya *et al.* (16, 23) made the significant discovery that enrichment with saprophytes yielded phages that attacked a variety of tubercle bacilli as well as saprophytes.

Hnatko (12) reported that 22 of 40 saprophytic strains exhibited sensitivity to one or more of 17 phages and could be arranged into nine tentative typing groups. The stability of the phages and the phage-sensitivity patterns were both stressed. Of the 26 phages isolated by Froman *et al.* (14), 22 were restricted in their host range to saprophytes and four acted on both pathogens and saprophytes. Employing these four, the authors found that 192 of 231 strains of tubercle bacilli, including human and bovine strains, were sensitive to one or more phages while 115 of 211 saprophytes were sensitive to the same phages. The sensitivity patterns of the two groups overlapped,

and the phages did not discriminate between virulent and avirulent tubercle bacilli. A potentially useful relationship (14) was observed in that all 14 strains of avian tubercle bacilli were resistant to these phages, as were 22 virulent and avirulent strains of *M. avium* studied by Will and co-workers (25). Takeya *et al.* (16) also noted that typical avian strains were resistant to phages capable of acting on human, bovine, and murine strains of tubercle bacilli. In further studies, Froman and colleagues (26) reported that while 40 of 48 strains, most of which were BCG, were sensitive to two or more of the four phages employed, there was no correlation between phage-sensitivity patterns and any other characteristic examined. Hnatko (27), using phages from various collections, found that most of the unclassified, non-pathogenic, acid-fast organisms isolated from patients admitted for investigation of tuberculosis exhibited a phage-susceptibility pattern characteristic for *M. smegmatis*. The study suggests that these organisms are widespread, having been isolated from sputum, urine, and material from gastric lavage. If similar patterns of phage sensitivity do not appear among the tubercle bacilli, these observations may be of some diagnostic value.

The following assessments seem reasonable on the basis of the preceding information. Despite the limited number of phages that have been isolated, a substantial percentage of both saprophytes and tubercle bacilli are susceptible to one or more phages, an important consideration in developing any typing scheme. Hnatko's (12) successful adaptation of four phages to new hosts points the way to further exploitation of available phages. There is no reason for abandoning the hope that phage may be useful in discriminating between or within groups of mycobacteria with some medical or diagnostic significance. An encouraging example is the insensitivity of avian tubercle bacilli to phages active on bovine and human strains.

The nature of mycophages.—The mycophages are morphologically similar to other phages. Electron micrographs show the characteristic head and tail (10, 12, 21, 28 to 31). In a few cases, the heads appear spherical but most are hexagonal, ranging from 50 to 80 mμ in width and 95 to 165 mμ in length. The tail lengths vary from 125 to 325 mμ and the tail width is 13 to 30 mμ. Penso (28) was one of the first to observe subunits in the head, which he interpreted as subunits of internal deoxyribonucleic acid, and he noted the structural inhomogeneity of the tail shaft and the bulbous nature of the tail tip.

Exclusive of morphology and host range, only a miscellaneous group of mycophage characteristics have been considered. Plaque variants were recognized and recombinant types may have been observed (11, 16, 32). In two instances phages were differentiated serologically (13, 16). Differences in heat sensitivity were reported (10, 11) but the experiments were inadequate and the results are only suggestive. The stability of mycophages to storage was cited in two instances (7, 13) and is implied from the absence of complaints by others. There has been no work on the chemical nature of the mycophages.

Mycophage reproduction: kinetics.—There is surprisingly little information about the one-step growth curve characteristics of mycophages (28, 30, 32 to 34). Latent periods range from 65 to 360 min., rise periods from 20 to 120 min., and burst sizes from 4 to 120. In total, very few data are available, much of what is known is incomplete, and standard methods have rarely been employed. These kinetic studies deserve more careful attention. One wonders, e.g., how the one-step growth curves of a given phage would compare when grown in a relatively fast-growing saprophyte as opposed to a slow-growing strain of tubercle bacillus.

Mycophage reproduction: adsorption and penetration.—Penso (28) described the tail-first adsorption of a mycophage to its host. In some cases, phage adsorbed at localized zones on the bacterial surface, suggesting an uneven distribution of receptor sites. The contraction of the phage particles and the loss of opacity of the head were observed after adsorption. Sellers *et al.* (29) failed to obtain electron micrographs of attached phage and, in an attempt to explain Penso's observations, suggested that surface tension forces during drying might have left phages lined up at the cell surface. However, the absence of adsorbed phage might be explained by Penso's observation (33) that after mixing phage and host cells adsorption began only after 40 min. had elapsed. The reason for this delay is not known, but some "conditioning" for adsorption is implied. Bowman (32) reported a calcium ion requirement for adsorption which varied in concentration both with the phage and the host. In some instances, abortive infections could also be partially overcome by calcium ion indicating a calcium requirement for penetration. Taken together, these results are consistent with an ion-dependent, tail-first adsorption followed by penetration of the phage head contents during a process involving the contraction of the phage. The similarity to the coliphage sequence is obvious.

It was shown by Groman & Bobb (36) that Tween 80 prevented the adsorption of a corynebacterial phage to its host cell. The widespread use of Tween in media for mycobacteria led White & Knight (37) to examine the effect of this non-ionic detergent on mycophage adsorption. Low concentrations of Tween 80 (0.01 per cent) prevented adsorption of Froman's D29 phage to a strain of *M. smegmatis*. Cells grown in Tween and washed free of it adsorbed phage normally. The addition of 10 to 25 per cent serum or serum albumin to the medium abolished the effect of Tween (38). The concentration relationships suggest that serum acts by binding Tween.

Mycophage reproduction: intracellular phase.—Aided by the fact that mycophage-infected cells become transparent to the electron beam, Penso (28, 33) first observed intracellular events in mycophage reproduction. He proposed a complex sequence of intracellular events including two distinct replicating stages for phage genetic material. This particular finding has not been corroborated by others (29, 31, 35). Fukai & Sellers (35) observed electron-dense bodies approximately the size of a phage head at a stage consonant with phage replication, and all authors have observed the so-

called "doughnut" forms which are probably empty phage heads. Although sectioned bacteria have been observed, a convincing picture of the morphological sequence of intracellular events is still lacking. Certainly, the published electron micrographs do not provide a suitable basis for judgment. In another study of intracellular events, Takeya *et al.* (34) observed changes in the chromatin of infected bacteria stained by HCl-Giemsa. Alterations in the chromatin arrangement were observed 10 to 20 min. after infection and culminated largely in "granular" cells with large ($>1\mu$) chromatinic bodies. This suggests synthesis of deoxyribonucleic acid during phage infection. There were no alterations in neotetrazolium-staining granules or metachromatic granules. While more specific evidence is awaited there is as yet no reason to hold, as Penso's formulation suggests, that mycophage reproduction differs fundamentally from that of the coliphages [see Kellenberger *et al.* (39)].

An interesting but unexplained phenomenon is the observation reported by White & Knight (40) that fresh human serum at concentrations of 10 per cent or greater decreased the yield of D29 mycophage in strains H37Rv and H37Ra of *M. tuberculosis* but not in that of a third strain, ATCC 607. The serum factor found in both normal and tuberculous individuals had the following properties: it did not prevent and may have enhanced phage adsorption, did not inactivate phage, was not diminished in activity by adsorption with either cells or phage, was stable under refrigeration and to 56°C. for one hour, precipitated in the serum globulin fraction, and was inactivated by trypsin. It is distinct from the serum albumin fraction which permits phage adsorption in the presence of Tween 80. It is possible that the factor described above does not interfere with the replication of D29 phage but rather enhances its readsorption to strains H37Rv and H37Ra. A second possibility is that the serum factor interacts with these strains or their extracellular products to release or produce a phage-inactivating agent.

Mycophage reproduction: lysis.—Nothing is known of the lytic process which is involved in the release of intracellular phage. The participation of lytic enzymes is suspected in other phage-host systems [Jacob & Fuerst (41)]. The halo of partial lysis (7) and "hot-cold" ring formation around plaques (11), suggest the action of a lysin. The possibility that lysins are produced is amenable to study and, indeed, the study of enzymes which would affect the cell wall structure of the mycobacteria would be of great interest.

Lysogeny in the mycobacteria.—It is almost an article of faith among phage workers that most, if not all bacterial genera, contain lysogenic organisms. To date, natural lysogeny has been demonstrated in only three strains of mycobacteria, none of which is a tubercle bacillus (cf. section on Source and Methods of Isolation). The success of the soil enrichment method suggests that lysogeny is more common than it appears to be, since these phages probably originated in lysogenic hosts. One possibility that has not been explored is that laboratory-imposed conditions are not optimum for the

demonstration of lysogeny. A study of soils which yield phage by enrichment might provide a clue to such an experimental defect. Another approach is to study known lysogenic organisms carefully. While existing systems may have been selected because of their ability to exhibit lysogeny under laboratory conditions, they may also represent fortunate accidents. Bowman & Redmond (30) studied growth and phage production by a lysogenic mycobacterium and demonstrated that the bacteria/phage ratio was very high—of the order of 10^6:1. The ultraviolet inducibility of this lysogenic strain was very low, with a maximum of 0.01 per cent induced, and the burst size was small. What ever the reason, this study and that of Segawa *et al.* (20) suggest that only small numbers of phage may be present in cultures of lysogenic mycobacteria, and this factor may contribute to the difficulty of demonstrating lysogeny.

The study of laboratory-produced lysogenic strains is another important source of information about the nature of lysogeny in mycobacteria. The feasibility of experimental lysogenization was demonstrated by Takeya *et al.* (16) and by Russell and his co-workers (19). The latter workers successfully lysogenized 14 of 35 strains of mycobacteria, employing seven different mycophages. The frequency of success varied with each phage. The criteria for lysogeny were adequate and the stability of the lysogenic state was established. What is puzzling is the successful lysogenization of strains of *M. tuberculosis* in the face of failure to demonstrate "natural" lysogeny in any strain of tubercle bacillus. Not only do these strains have the potential to be lysogenized but there is evidence that they carry other prophages. Russell *et al.* (22) reported that phage reisolated from lysogenized cells was different from the input phage. They listed host range, serological and plaque size differences, and an increased ability to lysogenize. These wholesale alterations indicate uncovering of, or perhaps recombination with, a phage already resident, and suggest once again that methods normally employed in testing for lysogeny may be inadequate.

Mycophage and mycobacterial variation.—Because phage plays a role ln bacterial variation through selection, transduction, and conversion it is natural to look for its effect on variation in mycobacteria. White & Knight (37) and Russell *et al.* (19) observed selection by phage of smooth variants of mycobacteria. The latter authors checked virulence and a number of biochemical characteristics of five non-lysogenic strains and their lysogenic derivatives and observed no differences. Takeya *et al.* (16) observed that strains with widely differing patterns of antibiotic resistance were similar in their phage-susceptibility pattern. In general, strains exhibiting flat, spreading, irregular, wrinkled colonies were less susceptible to phages than others. Many attenuated strains of tubercle bacilli exhibiting this colonial morphology were less susceptible than the virulent parents with rough, irregular, raised, and granular colonies. However, since H37Rv and H37Ra exhibited similar susceptibility patterns and similar colonial morphology, it was concluded that colonial morphology and not virulence correlated with

phage pattern. Juhasz (42) recently observed that streptomycin resistance could be transmitted by filtrates of a drug-resistant strain of BCG to a sensitive strain of *M. phlei*. Although transduction is a possibility, there is no evidence for the participation of phage as yet. An assessment of the role of phage in mycobacterial variation has only been initiated by these observations.

Phages of Hemolytic Streptococci

Sources, isolation, and relation to bacterial group and type.—Although a few suggestive reports preceded their work, Clark & Clark's (43) isolation of a streptococcal phage (streptophage) is generally accepted as the first. This phage was obtained from activated sludge enriched with *Streptococcus mucosus* isolated from a rabbit with a fatal septicemia. Similar efforts to obtain phages for other streptococci or *Diplococcus pneumoniae* strains failed, as did efforts to adapt the isolated phages to these strains. Shwartzman (44) adapted the Clarks' phage to a group of 16 of 21 hemolytic streptococci isolated from cases of erysipelas. The adapted phages were serologically related to the original one. This was a single success in a series of failures. No other strain among 102 human pathogens, including alpha, beta, and non-hemolytic streptococci, was susceptible to the Clarks' phage, nor were 64 strains of varying hemolytic character susceptible to the adapted phages. Lancefield's study of this phage (45) stressed its specificity. Of 119 strains in serologic groups A, B, C, D, and E, 47 of 49 group C strains and 3 group E strains were sensitive. Slight sensitivity of six group A strains was not ascribable to phage (46). All of the sensitive strains were of animal origin.

The most extensive work with streptophages was that done by Evans. In studies extending over a decade, sensitivity to phage and "nascent" phage was employed as an auxiliary test in defining groups of hemolytic streptococci. "Nascent" phage activity depended on the use of fresh lysates (cf. section on Lysis). Eventually, five serologically distinct phages were used; the Clarks' phage, one isolated from a case of intestinal hemorrhage (47), and three isolated from sewage (48, 49). Evans and co-workers (50 to 53) noted a number of correlations between phage-sensitivity patterns and the physiological and serological characteristics of the hemolytic streptococci. The correlation between phage sensitivity and serological group (49, 53) is still relevant. Recently, Krause (54) and Kjems (55) confirmed the specificity of group barriers with phages active on group A streptococci, and Kjems noted a similar group specificity for phages active on the enterococci. Both authors reported a limited type specificity for phage although Kjems could not detect a correlation between the amount of M antigen present and sensitivity to or capacity to produce phage.

In the interval between Evans' and Kjems' studies, the concept of lysogeny became firmly established, and it was natural for Kjems (55) to look for phages in the strains themselves. One hundred twenty-three group

A hemolytic streptococci were screened for phage production on an intra-T-type basis, and 11 enterococci were also screened on homologous strains. Four phages were isolated from and for the group A streptococci and four from and for the enterococci. Another estimate of the frequency of lysogeny among group A streptococci was made by Krause (54). Employing but two indicator strains he found that 12 of 52 strains, including three of five types screened, were lysogenic. Both the extent of lysogeny in beta-hemolytic streptococci and its relation to epidemiology are reflected in Kjems recent study (56). A total of 353 isolates from throat swabs of patients were examined. Of these, 217 came from a widely distributed (broad) group of patients while 136 were from patients in a single sanatorium. All isolates were typed for T antigen and tested for lysogeny with a single indicator strain. Despite this latter limitation, 100 isolates (28 per cent) were demonstrably lysogenic. Curiously, lysogeny was three times more frequent in isolates from children of the broad group than in the adults. Lysogenic strains were distributed among a variety of T types both in the broad group (9 of 20) and in the sanatorium group (6 of 18). While lysogeny was prominent in types 12 and 22 in the broad group, other types were well represented. In contrast, 90 per cent of all the lysogenic strains in the sanatorium group were type 12. It was later shown (57) that 49 of 52 phages from these type 12 organisms were serologically related. Of additional interest is the observation that during a four-month period in the sanatorium, non-lysogenic type 12 strains were displaced by their lysogenic counterpart. How did this occur? Nosocomial spread is clearly implied, but was this the result of simple displacement or the interaction of lysogenic and non-lysogenic strains in a single individual, or could phage alone have been transmitted to carriers of a non-lysogenic strain? Such questions have not been approached experimentally. Clearly, lysogeny is common among the beta-hemolytic streptococci and has potential significance and utility in epidemiological studies.

Streptophage reproduction: adsorption and penetration.—In studies of streptophage reproduction the various phases of the cycle have been related to specific products or components of the organisms, in particular, cell wall components. The net result is that much is known about the early and late phases and nothing about the intracellular phase.

Lancefield's observation (45) that strains producing mucoid colonies on blood agar were susceptible to a group C phage suggested that hyaluronic acid might be a factor in phage-host interaction. Maxted (58) observed a reverse relationship in that mucoid colony formers among the group A streptococci were phage-resistant, whereas glossy, non-hyaluronic acid-producing variants were phage-susceptible. Resistance of the mucoid strain could be abolished by hyaluronidase treatment. Thus, the hyaluronic acid capsule acted as a barrier to adsorption of phage, but once the capsule was removed the strain was susceptible. Extending his original work, Maxted (59) observed that resistant mucoid clones appeared following strong phage action on 36 of 50 non-mucoid group A streptococci. A suggestively similar

observation was made by Kjems (55), and Krause (54) fully confirmed the relationship between capsule production and resistance to Maxted's phages. Kjems' finding (60) that one of two phages studied was active on encapsulated group A strains showed that this barrier to phage infection could be overcome naturally. How was this accomplished?

The first clue was Kjems' observation (60) that the enzyme hyaluronidase appeared in lysates following propagation of certain phages on non-hyaluronidase-producing strains. Strains lysogenized by this phage did not produce the enzyme, thus linking its production to the lytic cycle. Furthermore, serologically distinct hyaluronidases were produced when the same strain was infected with either of two different phages [Kjems (60)], indicating that the enzymes were phage-induced and not simply enzymes of the host released by lysis. Finally, three serologically distinct hyaluronidases representing combinations of three types of group A streptococci and three serologically distinct phages were identified in lysates. The serological grouping of the enzymes correlated with the serological grouping of the phages.

The relation of phage-induced hyaluronidase to phage constituents was also investigated by Kjems (61). Following ultracentrifugation, both hyaluronidase and phage titers were significantly reduced in the supernatant. Even more enzyme activity was removed by absorbing the lysate with the propagating strain. Therefore, much of the enzyme is adsorbed to or is an integral part of the phage. The parallel between phage-inactivating ability and antihyaluronidase activity of sera prepared to phage lysates certainly supports this idea [Kjems (60)]. This close physical relationship of phage and enzyme appears to explain the ability of these phages to penetrate the capsule of hyaluronic acid-producing strains.

The distribution and epidemiological significance of phage-induced hyaluronidases is of obvious interest. Kjems (56) stated that antibodies to these hyaluronidases were found in sera submitted for antistreptolysin O and antistreptococcal hyaluronidase determinations. In this preliminary study, Kjems grouped 88 streptophages into four serological groups which in turn correlated with the type of hyaluronidase produced. It seems uncertain to this reviewer whether the phage and the hyaluronidase groups are actually based on different antigenic entities, but in any event their number is low enough to permit a study of antihyaluronidase activity in patients' sera. Interestingly, Kjems (61) found that three phage-induced hyaluronidases proved insensitive to a patient's serum with a high antistreptococcal hyaluronidase titer indicating that still another serologically distinct enzyme exists. Kjems distinguishes between phage-induced and streptococcal, i.e., bacterial hyaluronidase, but the validity of this distinction is open to question. In view of all these findings, the statement that the hyaluronidases of streptococci are group- but not type-specific (62) must now be reconsidered.

In addition to the influence of hyaluronic acid and hyaluronidase of phage adsorption, some effort has been made to characterize phage receptor material in relation to other streptococcal antigens. Krause (54) found that

a group C-specific phage was inactivated by the cell walls or the extracted group carbohydrate of two group C organisms, whereas this material had no effect on group A phages. Extracted group A carbohydrate did not inactivate either phage type although both phages adsorbed to it reversibly. Thus, group C carbohydrate functions as phage receptor while the A substance, which differs physically and chemically (63), does not. A similar conclusion was also reached by Kowalska & Pakula (64). Nevertheless, Krause (54) has observed that group A substance is released from cell walls exposed to phage and that the morphology of the cell walls as determined by electron microscopy was altered. Some correlation has been noted (54, 55) between phage sensitivity and the M or T type specificity of streptococci, but in neither instance is there any evidence that the type antigens participate in adsorption.

Streptophage reproduction-lysis.—Literally nothing is known about the reproduction of phages active on beta-hemolytic streptococci between adsorption and release. Observations pertinent to the lytic phase date from the work of Evans (65) on "nascent" phage. The basic observation was that during lysis of a susceptible strain, collateral lysis of a phage-resistant streptococcus would also take place. Freshly prepared lysates were also active, hence the term "nascent" phage or "nascent" lysis. Unrelated strains, e.g., *Diplococcus pneumoniae* and *Staphylococcus* sp., were also lysed in this manner, emphasizing that lysis was not caused by phage reproduction in the resistant strains. Evans (66) recognized that a lytic enzyme might be released during lysis of the susceptible strain.

The study of nascent lysis in streptococci was revived simultaneously by Krause (54) and by Maxted (67). Fresh lysates prepared on group C streptococci lysed viable, phage-resistant group A, E and H bacteria as well as related group C strains. The lytic factor was produced whenever phage lysis ensued, regardless of the host or phage employed, but Evans (65) found that with a given phage the range of activity of a lysate varied with the propagating strain. Krause's findings (63) indicated that some of the lytic activity was phage-bound, but Maxted (67) found that the active lysin was not removed by ultracentrifugation, as was phage, suggesting that most of it was free. Lysin was active at pH 6.5 to 8.6 but was destroyed by extremes of pH. Its activity was lost on standing but was restored by thioglycollate. It was destroyed by proteolytic enzymes and was precipitated by 40 per cent saturated ammonium sulfate. Despite this evidence of its protein nature, antilysin antibodies could not be demonstrated in a serum containing phage-neutralizing antibodies. Lysin did not attack cells heated to 56°C. or 85°C. unless they were pretreated with proteolytic enzymes. This suggested that lysis required the cooperation of a proteolytic enzyme. A search failed to reveal such an enzyme in active lysates.

There is both chemical and visual evidence that lysins produced in streptococci act on cell wall constituents. Maxted (67) noted the release of group-specific polysaccharides during lysin action on intact cells. Krause

(54, 63) made similar observations during lysis of cell wall preparations, and showed that the lysin removed group-specific carbohydrate almost quantitatively. Type-specific M protein was also released during lysin action, and this observation provided Kantor & Cole (68) with a less drastic method for isolating M protein than boiling cells at pH 2. The similarity between the products of phage lysis and those produced by lysin action suggests an obvious relationship between the two events.

Lysin activity has been observed in other phage-host systems [see (67)] but so much is known about the antigenic nature of streptococci that they are particularly valuable for studying the chemistry of lysin action. As in other systems, the evidence indicates that streptococcal lysin is not present in uninfected cells and therefore is probably phage-induced.

Streptophage: miscellaneous information.—As was already pointed out, there is a paucity of information on the intracellular phases of phage reproduction, and this paucity extends to other areas as well. Kjems (60) reported latent and rise periods ranging from 55 to 61 min. and 10 to 19 min., respectively, and a burst size of 32 for one phage. The size of one streptophage was given (61); head, 45×39 mμ and tail 177 mμ long. Serological distinctions were reported (49, 65) between phages attacking different groups of streptococci as well as phages attacking different types within one group (61). It is encouraging for future work that 88 group A phages could be classified into four serological groups (57). Streptophages vary in heat stability (60, 65) but appear to be fairly stable on storage (57, 65). Variation in plaque size and morphology have been noted [e.g. (55, 65)] but a systematic study of these characteristics is lacking. It seems obvious that much additional information about streptophages is needed. The potential significance of phage-host relationships among the beta-hemolytic streptococci to streptococcal disease to be discussed next, should provide a focal point for further study.

Streptophage and streptococcal variation and virulence.—Early studies of streptococcal phages centered on their potential usefulness as therapeutic agents and as aids in the preparation of more effective vaccines. On the basis of limited observations, Dutton (69) suggested that patients with streptococcal infection recovered spontaneously when the infecting strain was phage-sensitive, as evidenced by lysis on the isolation plates. However, contrary to hope, the inoculation of infected animals with streptophages appeared to be detrimental (70 to 72). The presence of toxic substances in the phage lysate was one explanation offered by Clark & Clark (70) for failure of phage therapy, and Evans' (72) observation that streptophage was inhibited by blood, pus, ascitic fluid, bile, saliva, and lysed red and white corpuscles was offered as a second. Maxted's finding (58) that serum enhances hyaluronic acid production suggests another way in which a non-hyaluronidase-inducing phage might be thwarted. In addition to its therapeutic potential, phage lysis as a means of producing effective soluble antigens for immunization, was also recognized. However, Evans (73) found phage lysates inferior to other preparations in providing protection for

rabbits and mice or in producing agglutinins. It is interesting to note that Jungeblut & Schultz (74) showed that lysates contained antigens differing from those obtained from uninfected bacteria.

The role of phage in streptococcal variation is as yet poorly defined. Selection of mutants by phage may account for Grumbach's observations on streptococcal dissociation (75), but Shwartzman's finding (76) that antigenic variations appear under the influence of phage are not easily explained in this way. The recovery of mucoid variants following phage action on a non-mucoid strain [Maxted (59)] is clearly an effect produced by phage, but neither fluctuation tests nor indirect replica planting permitted a decision between selection or induction of a mucoid variant by phage. Although the former mechanism was favored by the author, calculation of the rate of appearance of mucoid clones strongly suggests a phage-induced change. It is significant from the viewpoint of virulence that the amount of M antigen increased in 9 of 10 mucoid variants. All strains surviving phage action were enhanced in virulence when tested for mouse virulence and ability to grow in human blood. It was noted that an increase in either hyaluronic acid or M protein enhanced virulence. This report of the selection of a more virulent organism by a method other than animal passage is unusual.

The isolation of some unusual variants following phage action was reported by Pakula & Walczak (77). The polysaccharide of one phage-resistant variant derived from a group A streptococcus was chemically and antigenically similar to group C polysaccharide. Two other variants had become alpha-hemolytic. The latter strains did not react with either group-specific A or C antiserum, and rahmnose and glucosamine were replaced by galactose as polysaccharide components. Apparently these variants were not lysogenic, but the authors left unsettled the question of whether selection or induction by phage was involved in the modifications.

Madison (78) made an interesting observation concerning fibrinolysin (streptokinase) levels and phage activity. Fibrinolysin levels of a phage-infected culture paralleled those of an uninfected culture until lysis occurred. During the lytic period, fibrinolysin titers dropped but rose again as phage-resistant growth appeared, and eventually attained a level ten times greater than that produced by the uninfected strain. Further study revealed that while fibrinolysin levels of the parent strain rose to a peak comparable to those attained with the phage-resistant mutant, they dropped sharply later in the growth cycle. In contrast, the mutant sustained the peak level. There was no evidence given to indicate whether phage acted selectively or played a causal role in altered fibrinolysin activity.

From time to time there has been speculation that a viral agent participates in scarlet fever. The observations of Frobisher & Brown (79) are of particular interest on this point. Using doubly marked bacterial strains, these authors were able to transfer the ability to produce erythrogenic toxin to a non-toxin-producing strain by a Berkefeld filtrate of a toxin producer.

The fact that isolates of newly produced toxin formers eventually lost this ability probably discouraged further study. Similar experiences were summarized by Bingle (80) who suggested that phage from toxin-producing strains induced toxin formation in non-toxin-producing strains. Curiously enough, no effort was made to demonstrate phage in active preparations. All of the observations made by Frobisher and Brown could be explained in the context of known phage-host relationships and are so suggestive they warrant reinvestigation. The parallel suggested by the relationship of diphtheria phage to diphtheria toxin is obvious.

Staphylococcal Phages

A comprehensive treatment of the staphylococcal phages (staphylophages) was originally planned as a part of this review. Approximatley 250 references were collected with this purpose in mind. Fortunately for this reviewer, the need for comprehensive coverage has been filled by a chapter in Elek's recent book (81). However, after reading a selection of about one-third of the references, differences in emphasis and organization became apparent and a résumé reflecting these differences and including more recent findings was considered worthwhile. The following, then, is not intended to be complete in the sense of citing all references or detail, but in the reviewer's judgment describes this group of phages. Where nothing can be added by reiteration of fact, reference will be made to Elek's chapter. It should also be consulted for additional references.

The nature of staphylophages.—The morphology of the staphylophages is similar to that of other phages. The phage consists of an hexagonal head and a long tail with a terminal "bob." A complexity of structure similar to that of coliphage is suggested by the studies of Hotchin and co-workers (82, 83). They observed head membranes, noted 10 to 20 mμ granules in the phage head, saw tail fragments and tail cores in purified preparations, and speculated that the tail consisted of a hollow cylindrical sheath containing a solid central rod. Seto *et al.* (84), employing the freeze-drying technique, described two groups of serologically distinct phages, one composed of particles with elongated hexagonal heads (36$\times$80 mμ) and long tails (15$\times$270 mμ), and a second of particles with regular hexagonal heads (53$\times$53 mμ) and shorter tails (10$\times$160 mμ). This same dichotomy, which would be interesting to investigate chemically, was also observed by Ortel (85). There is, in fact, very little information on the chemical nature of staphylophages. Their antigenicity (86, 87) implies protein constituents, and the only indication of nucleic acid is Hotchin's observation (83) that supernates from osmotically shocked phage showed a nucleic acid adsorption spectrum.

As reviewed by Elek (81), staphylophages are sensitive to a variety of physical and chemical agents and to extracts from certain sensitive and resistant bacteria. No unusual attributes are exhibited. Their varying sensitivity to storage at refrigerator temperatures (86) and to chloroform (88) suggests caution in the method used to preserve a particular phage. The

most recent addition to the list of agents inactivating staphylophages is a rabbit serum component the activity of which is destroyed by zymosan (89).

Staphylophage reproduction: general observations.—The most representative data for the kinetics of staphylophage reproduction are found in Rountree's study of typing phages (90). Depending on the phage, latent periods varied from 35 to 60 min. and lysis was complete at 55 to 75 min. Burst sizes for 20 phages varied form 10 to 140 particles under optimal conditions. Plaque sizes varied from 0.3 to 1.2 mm. (86).

Staphylophage reproduction: adsorption and penetration.—The adsorption of a staphylophage to its host, tail first, follows the standard pattern (82). Rountree (90, 91) reported that specific electrolyte concentrations, particularly calcium ion, were critical for adsorption of all but a few of the 23 phages studied, and in some instances calcium was also required for penetration.

Burnet & Lush (92) and Freeman (93) isolated a phage-inactivating polysaccharide from sensitive staphylococci. Rountree (94) isolated a phage-inactivating material which she tentatively identified as nucleoprotein. In both cases, phage-receptor substance was probably involved. Receptors for a given phage appear to be widely distributed among the staphylococci including phage-resistant strains. Rountree, e.g., found that certain phages which lysed some of the coagulase-positive strains tested adsorbed to all of them. The specificity implied in this observation does not always hold since Rountree (86) has shown that phage K adsorbed to and reproduced in both coagulase-positive and negative strains. Rakieten and co-workers (95, 96) showed that staphylophages adsorbed to such unrelated species as *Bacillus subtilis* and enterococcic streptococci. Staphylophage-inactivating material was actually extracted from the enterococci and the adsorption of enterococcal phages on staphylococci was also demonstrated. Such observations are generally explained on the basis of the accidental occurrence of a common antigen, but with the methods available for infecting normally resistant bacteria with bacteriophage [e.g., Fraser, Mahler *et al.* (97)], these relationships could be put to more stringent tests.

It is a general observation that antibody to a bacterial strain can block phage adsorption, presumably by blocking the receptor sites. The bacterial surface may also be involved in the observation reported by Beumer-Jochmans (98) that sensitive virulent but not avirulent staphylococci were protected from phage action by a heat resistant pseudoglobulin found only in normal sera of rabbits and other animals. One final observation, probably related to the initial interaction of phage and host, is the bactericidal effect of high concentrations of certain staphylophages on resistant hosts as observed by Beumer-Jochmans (99, 100). The high phage: bacteria multiplicity (100:1) required for this effect suggests a phenomenon similar to lysis-from-without, but apparently lysis did not accompany cell death in this case. In view of the requirements for staphylococcal lysis, this is not too surprising (cf. section on Lysis).

Staphylophage reproduction: intracellular phase.—The relation of phage

reproduction to bacterial reproduction received much attention in the early work of Krueger and his associates. Ultimately it was shown that conditions permitting bacterial reproduction were not a prerequisite for phage reproduction (101, 102). Still unexplained is the observation of Krueger *et al.* (103) that a significant increase in phage activity was noted within 5 min. after infecting log phase cells recently suspended in Locke's solution at 5°C. A short exposure of the cells to 45° to 50°C. abolished their ability to permit this increase. Clearly, this increase in phage activity did not involve reproduction. A simple explanation, such as the unmasking of phage bound by an inhibitor comes to mind, but an experimental answer has not been published. As a part of their over-all program, Krueger and associates also studied the influence of temperature on phage reproduction, a topic pertinent to recent work in virology. They found (104) that little or no increase in phage could be detected at 42.3°C., and that at 47.5°C. a high percentage of infected cells were actually "cured" of their infection (105). The depressing effect of elevated temperature (44°C.) on staphylophage yield was also observed by Beumer-Jochmans (99). Neither thermal inactivation of the host nor a depression of its reproductive capacity was involved.

There have been a number of investigations on the biochemistry of staphylophage infections. The most extensive work was done by Price with a phage isolated from the common housefly [cf. Elek (81); Price (106)]. In summary, these studies revealed a shift in nucleic acid synthesis following phage infection. Deoxyribonucleic acid synthesis was favored although ribonucleic acid synthesis continued during phage reproduction. Protein synthesis continued in excess of the needs for phage replication. This general description follows the pattern for coliphages, but more particularly for temperate phage which, in contrast to vlrulent phage, permits net ribonucleic acid syntheiss during its reproduction (107). In other studies, Price (108, 109) observed that there were specific nutritional and metabolic requirements for phage reproduction.

Chemical interference with staphylophage reproduction has also been studied to some extent. A number of investigators observed that acridine compounds at sub-bacteriostatic levels inhibited-phage reproduction without impairing the lytic mechanism (110 to 112). Hotchin's evidence (111) is consonant with the idea that acridines inhibit a very late stage in the reproductive process as they do with coliphages. The effect of antibiotics on phage reproduction has also been investigated. By far most of the work, to be dealt with in the next section concerns penicillin. However, a series of studies by Edlinger and Faguet [cf. Elek (81)] showed that under appropriate conditions streptomycin, chloromycetin, and aureomycin interfered with staphylophage reproduction.

An investigation by Rountree (113) on the fate of infecting phage antigen and the timing of the intracellular appearance of two phage antigens, presaged much present day work. She showed that one of the phage antigens remained external to the cell following infection, and that a complement-

fixing antigen appeared intracellularly long after infection began and reached its peak before phage maturation occurred. These observations reflected the separation of protein and nucleic acid after adsorption, suggested the antigenic complexity of phage, and revealed the possibilities of kinetic studies of intracellular events.

More work has been done with staphylophage reproduction than with many other phages. Even so, the biochemical, cytological, and genetic approaches have yet to be systematically applied. Still, it is important to note that the significant observations with staphylophages make sense in the context of our understanding of coliphages.

Staphylophage reproduction: lysis.—The lytic step of the staphylophage cycle has probably received as much attention as any other aspect of the reproductive cycle. Eisenberg-Merlin (114) observed with dark ground illumination that staphylophage release was accompanied by a slow disintegration of the coccus in contrast to the explosive breakdown of lysing coli bacilli. Farrant & Rountree (115), who used electron microscopy, commented that lysed cocci appeared to have a hole in only one spot. They felt that this less destructive lysis might be related to the slow decrease in optical density during phage release, an observation also made by Goffart-Roskam (116). Slow clearing of an infected culture was usually associated with a low yield of phage. Related to these findings, although in seeming contradiction, is Hotchin's observation (117) that phage yields were increased and lysis was slower if 5 per cent (w/v) of ammonium sulphate was added to cultures within a few minutes after infection. Most puzzling was his observation that in one-step growth experiments burst size was not increased by the salt. The paradox of an increased yield in mass culture and no increase per cell in diluted cultures was not resolved.

A hypothetical explanation of these observations relating to staphylophage lysis now appears possible on the basis of some excellent work by Ralston and her associates. An historical digression is required first. Wollman & Wollman (118) and Rakieten (119) both reported that a phage-resistant staphylococcus could be lysed if mixed with phage and a strain sensitive to it. Similarly, Welsch (120, 121) observed collateral lysis of unirradiated intact cells when mixed with ultraviolet-induced cells and kept at temperatures close to 0°C. All of these investigators recognized that something other than phage was produced during the reproductive cycle which secondarily led to lysis of the uninfected component of the system. It was, in fact, the lysins produced by staphylococci, virolysin and autolysin, which Ralston *et al.* (122, 123) studied. Virolysin is phage-induced, i.e., produced only as a consequence of phage infection, and was distinguished on serological and operational criteria from autolysin which can be produced by holding cells at 4°C. for one to five days. Both lysins are proteins, behave as enzymes, and appear to act on the bacterial cell wall. Both lyse heat-killed cells but cannot attack viable log phase cells. The final, critical bit of information on phage lysis of staphylococci was supplied by Ralston and her co-workers

(124). They established that a sensitization step preceded lysis by virolysin, and that, if sensitized, log phase cells could also be lysed. The method of sensitization pertinent here was the adsorption of an average of five viable or ultraviolet-inactivated phage particles per cell. Sensitization was successful at both 4°C. and 37°C.

An interpretation of the facts relating to lysis by staphylophage is now possible. When a cell releases phage, by whatever mechanism is involved, it can be assumed that virolysin is also released. Virolysin will itself not attack other cells and perhaps not even cell walls unless there is sufficient free phage present for sensitization. If phage yield is low then, of course, sensitization will be limited and clearing will be slow. If phage yields are high but readsorption of phage is prevented as, e.g., by ammonium sulfate, then collateral lysis which requires both virolysin and phage is prevented and cells which would have been lysed before acquiring a full complement of phage, are permitted to produce full bursts. If, in such a system, dilution occurred shortly after infection, then salt would not influence average burst size. In a diluted system sensitization would not occur and, in addition, virolysin concentration would probably be too low to be effective. The paradox posed by Hotchin's observations (117), namely, of increased phage yield in mass cultures in the presence of salt with no parallel increase in phage yield per cell in diluted cultures is thus resolved.

All observations in which either phage-resistant or sensitive cells are secondarily lysed become understandable on the basis of virolysin action alone or its combined action with phage. As previously indicated, phage receptors are widespread among the staphylococci, including phage-resistant organisms. Steps associated with adsorption or penetration, or both, may be sufficient to sensitize a cell to virolysin action. In some cases, sensitization may not be required or may occur by other means. Ralston *et al.* (124), e.g., reported that treatment with penicillin also sensitized, and indeed the numerous reports of a synergistic action between phage and penicillin on lysis time (102, 125, 126) find a reasonable explanation in the penicillin sensitization of cells. It is not known what sensitization is, but it appears to involve alteration of the cell wall so as to make virolysin action possible.

Lysogeny in staphylococci.—Lysogeny is common among the coagulase-positive strains of *S. aureus* isolated from human sources, ranging from 44 to 90 per cent (127 to 130). The phenomena associated with lysogeny parallel those known for the enteric bacteria [cf. Bertani (131)]. Lysogeny has been produced experimentally by a number of workers (132 to 136) although almost nothing is known about the factors with determine whether lysogenization or lysis will occur. Multiple or polylysogeny, in which one strain may carry a number of serologically related or unrelated phages, has also been reported (86, 137, 138).

One of the cardinal features of lysogeny is the immunity of the host cell to super infection by carried phage and often closely related phages. Lysogeny may also confer resistance to serologically unrelated phages previously

able to reproduce on the host. Most surprising is the finding that following lysogenization bacteria may acquire sensitivity to phages previously resisted. All of these phenomena have been observed in staphylococci by investigators concerned primarily with a change in phage-typing pattern (135, 136, 139). Extensions of immunity to homologous or heterologous phages following lysogenization are simply understood on the basis of the newly established immunity. Gains in sensitivity following lysogenization have a more varied interpretation. Gorrill (134) concluded that the substitution of one prophage for another with a consequent alteration of the immunity pattern explained the gain in sensitivity. Rountree (136) proposed that such changes resulted from the selection by phage of pre-existing mutants with the new sensitivity pattern. Asheshov & Rippon (135), investigating a similar situation, have excluded lysogenic conversion, transduction, and prophage substitution as potential mechanisms. They doubt the possibility of mutant selection and state there is no evidence to show that defective prophage is involved. They do not know the mechanism in their example but think that lysogenization is an important element.

The lysogenic state is reversible. Under appropriate conditions, induction is usually followed by lysis and phage production. Our knowledge of factors affecting induction of lysogenic staphylococci comes largely from the efforts of Welsch and co-workers (129). These workers showed that all the lysogenic strains they studied exhibited the "Lwoff" effect, i.e., induction by ultraviolet light. Sodium thioglycollate at $M/30$ to $M/80$ was also an inducer. A short, 15 to 120-sec. thermal shock at 55 to 60°C. followed by rapid cooling to 37°C. also caused some induction, an observation for which the reviewer knows no parallel among enterophages. Welsch *et al.* have also defined certain physiological factors affecting induction and ultimate release of phage. Most important is the observation that phage synthesis would not occur unless a prelytic incubation period under conditions of growth was permitted. Treatment of induced cells with bacteriostatic agents during the first half of the prelytic period suspended the induction process for as long as 24 hr. Progressive heating to 50°C. or exposure to light of longer wavelength either before or soon after ultraviolet irradiation, exerted either a prophylactic or curative effect on induction, but the strain remained lysogenic. Actual curing of a lysogenic strain of its phage was accomplished by Huybers & Jennings (140) by heavy ultraviolet irradiation. The frequency of such cures in this and other systems is low and the mechanism is still unknown. That loss of a carried phage is not inevitably accompanied by cell death, is also shown by observations of prophage substitution.

In summary, it is clear that lysogeny among the staphylococci is common and similar in its outlines to the phenomenon in enterophages. Direct proof or suggestive evidence is available for polylysogeny, homologous and heterologous immunity, prophage substitution, prophage cure, induction, and defective prophage. As yet, no single major feature sets lysogeny among the staphylococci apart from that found in other systems.

Staphylophage and staphylococcal variation.—Host-induced phenotypic variation in staphylophages was discovered by Ralston & Krueger (141, 142) simultaneously with its discovery in the enterophages. The relative plating efficiency of a given phage on two hosts was dramatically altered following a single reproductive cycle in either of two hosts. The phenotypic nature of the alteration and the function of the propagating host in this alteration were clearly established. Additional observations of host-induced modifications were indicated in the work of Gorrill (134) and Rountree (139). Of particular interest in the former work, is that temperateness or virulence of the phage appeared to be host-controlled.

The effect of staphylophage on the variability of staphylococci has a number of facets. The most obvious indirect effect is the selection of phage-resistant mutants. Resistant *S. albus* clones with altered colonial morphology were noted by Burnet & McKie (143). Wahl (144) observed unusual changes in phage-resistant growth of *S. albus* which strongly suggests L form production. Staphylophages also exert direct effects on the characteristics of staphylococcal strains through the mechanisms of conversion and transduction. As indicated in the previous section, the development of new and heritable phage-typing patterns was the direct consequence of lysogenization. The ability to produce staphylococcal virolysin (cf. section on Lysis) is also causally related to the presence of phage. It seems safe to predict that other traits of staphylococci will eventually be related to the phages they carry. In this connection, Larkum's observations (145, 146) suggesting that a phage component might be serologically related to dermonecrotic toxin is worth consideration. In addition to conversion, Cavallo & Terranova (147) and Morse (148) have produced evidence pointing to transduction of drug resistance markers by staphylophages. Morse found that streptomycin or novobiocin resistance was transferred at a rate proportional to the phage concentration by approximately 1 in 10^7 to 1 in 10^8 plaque-forming particles derived from a drug-resistant host. Of the 462 transduced strains, 461 were lysogenic and the other was phage-resistant, suggesting a defective prophage. High-frequency transducing phage preparations could not be prepared from transduced strains. Fermentation markers were not transduced at a measurable frequency. Parenthetically, efforts to demonstrate recombination between appropriately marked strains also failed. The potential significance of the various mechanisms of genetic transfer to such problems as acquired drug resistance, or the epidemiological relationship of staphylococci is very great.

With the advent of phage typing of coagulase-positive staphylococci, many attempts have been made to correlate phage type with other characteristics of staphylococci. As reviewed by Elek (81), there is no apparent relationship of phage type to such characteristics as alpha- or beta-hemolysin, fibrinolysin, or pigment production. More recently, Oeding & Williams (149) reported that in only a few cases were phage type and serotype closely correlated. There is a tendency for drug resistance to appear more frequently

in strains belonging to phage typing group III, and there is good indication that enterotoxin production is characteristic of some organisms belonging to this group. Barber & Wildy (150) found that three serologically distinct coagulases were closely correlated with three phage typing groups, and recently Faber & Rosendal (151) reported some correlation between some phage types and the amount of hyaluronidase produced.

What does it mean if one finds that certain characteristics of bacteria are correlated with their phage type? Of course, such correlations suggest that these groups of bacteria have certain common biological denominators which, at first sight, are reflected in their ability to reproduce a set of phages. Specifically, one would like to know if there is a link between these events whose description in chemical and biological terms would permit a better understanding of the correlation. There is no simple answer. In general, phage activity may be involved in one of two ways. First, one can attempt to relate the correlated bacterial trait directly to some accessible step of the lytic cycle of the typing phages, e.g., adsorption, penetration, vegetative reproduction, lysis etc., and in some instances meaningful relationships will emerge. Second, since typing phage activity may be a reflection of phages already carried by the bacteria, one may attempt to relate the correlated bacterial trait to the carried phage and perhaps uncover a secondary phage-based relationship. Certainly, the chances of success in either case are unpredictable. They become improved as the correlation is strengthened and as the group with a given phage type and a particular trait contracts.

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PLANT-NEMATODE INTER-RELATIONSHIPS[1]

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Introduction

This review is restricted to the plant-parasitic nematodes. They are certainly not the only nematodes whose existence has some relation to plant growth. It has been suggested that rhabditoid species, which normally feed on bacteria, sometimes "nibble" at root hairs or at young tissue in buds, and damage plants by devouring bits of tissue. The same may be done by some dorylaimoid species with wide hollow stylets or teeth. The abundance of bacteria, fungi, and small animals which constitute the food of many other nematode species, often depends entirely on plant growth. The relationships between plants and these nematodes are much more facultative and remote, however, than are those between plants and plant-parasitic nematodes.

Plant-parasitic nematodes feed on the liquid contents of plant cells by means of hollow stylets whose lumen is too narrow even for bacteria. Most of these nematodes belong to the order *Tylenchida*, but a few species belong to the quite different superfamily *Dorylaimoidea*. They are all obligatory parasites and feed only on plant hosts comprised of fungi, thallophytes, bryophytes, pteridophytes, and higher plants. Several nematode species have been found or are suspected to live on fungi only: *Ditylenchus triformis* (64), *Tylenchus*, and *Psilenchus*. Others live on both fungi and higher plants: *Ditylenchus destructor* (3), *D. myceliophagus* (133), *Aphelenchoides besseyi*, and *A. fragariae* (23). Many species seem to feed on higher plants only.

Classification of Plant Parasitic Nematodes Based Upon Their Parasitic Behaviour

The simplest ecological classification of the plant-parasitic nematodes is based upon the part of the plant which is attacked: (*a*) leaf and bud nematodes (*Aphelenchoides ritzemabosi, A. fragariae, A. besseyi, A. blastophthorus*); (*b*) stem and bulb nematodes (*Ditylenchus dipsaci, D. destructor*); *Aphelenchoides subtenuis*, which species is generally not included in this group, attacks bulbs and corms in much the same way as does *D. destructor;* (*c*) stem and seed gall nematodes (*Ditylenchus graminophila* etc., *Anguina* species; (*d*) root nematodes.

The group of the root nematodes is very large and needs further differentiating, which is mostly done in terms of conspicuous symptoms or stages, e.g., root knot nematodes (*Meloidogyne* spp.), cyst-forming nematodes (*Heterodera* spp.), and root lesion nematodes (*Pratylenchus* spp.). There are two other categorical distinctions: (*a*) ectoparasitic species which,

[1] The survey of the literature pertaining to this review was concluded in November, 1960.

TABLE I.

FEEDING HABITS OF ROOT NEMATODES AND REACTIONS OF INVADED TISSUE

		Part of plant invaded	Tissue on which nematodes feed	Nematode species	Reaction of plants: Essential to feeding	Reaction of plants: Resulting from feeding	References
Migratory	ectoparasitic	elongation zone of root	root hairs root epidermis	*Tylenchorhynchus*, *Tetylenchus*	unknown unknown	reduction of root system	(51, 80, 82)
		root epidermis	root epidermis	larvae of *Cacopaurus*	unknown	necrosis	(143)
		root cortex	parenchyma	*Belonolaimus*	unknown	necrosis	(22)
				Criconemoides	unknown		
				Dolichodorus			
				Longidorus	unknown	necrosis	(67)
				Paratylenchus			
				Xiphinema	unknown	galling, necrosis	(125)
				larvae of *Nacobbus serendipiticus* (?)	unknown	necrosis	(41)
		root	epidermis and cortical parenchyma	larvae of *Sphaeronema*	unknown		
				larvae of *Trophotylenchus*	unknown		
				larvae of *Tylenchulus*	unknown		(149)
		root tips	apical meristem	*Belonolaimus*	unknown	inhibition of mitosis in apical meristem (stubby root) necrosis	(22)
				Dolichodorus	unknown	inhibition of mitosis in apical meristem (stubby root) necrosis	(117)
				Hemicycliophora	unknown	inhibition of mitosis in apical meristem (stubby root) necrosis	(148, 150)
				Trichodorus	unknown	inhibition of mitosis in apical meristem (stubby root) necrosis	(24)
	semiendoparasitic	root cortex	parenchyma	*H. uniformis*	unknown	necrosis	(131)
				Helicotylenchus	unknown	necrosis	
				Pratylenchus (on thin roots)	unknown	necrosis	(80, 101)

TABLE I. (*Continued*)

		Part of plant invaded	Tissue on which nematodes feed	Nematode species	Reaction of plants: Essential to feeding	Reaction of plants: Resulting from feeding	References
Migratory	semiendoparasitic			*Rotylenchus buxophilus*	unknown	necrosis	(46)
				Tylenchorhynchus macrurus	unknown	necrosis	(47)
		root cortex	phloem parenchyma and elements	*Hoplolaimus coronatus*	unknown	necrosis of phloem and xylem	(85)
	endoparasitic	root cortex	parenchyma	*Pratylenchus*	unknown	necrosis; root rot	(56)
				Radopholus	unknown	necrosis; root rot (hypertrophy under sterile conditions)	(25, 32)
				Scutellonema	unknown	necrosis	(141)
				larvae of *Nacobbus*	unknown		(144)
			phloem	*Hoplolaimus coronatus*	unknown	necrosis	(85)
Sedentary	ectoparasitic	root cortex	epidermis	♀♀ of *Cacopaurus*	unknown	necrosis	(143)
	semiendoparasitic	root cortex	pericycle? vascular elements?	♀♀ of *Rotylenchulus*	unknown	necrosis	(94)
				♀♀ of *Sphaeronema*	unknown	necrosis	(122)
				♀♀ of *Trophotylenchus*	unknown	necrosis	
				♀♀ of *Tylenchulus*	unknown	necrosis	(149)
	endoparasitic	just behind root tip?	vascular elements?	♀♀ of *Nacobbus*	?	hypertrophy of invaded tissue	(41, 144)
				♀♀ of *Meloidodera*	?		(14)
		just behind root tip	vascular elements	♀♀ of *Meloidogyne*	formation of giant cells	hypertrophy of invaded tissue	(81)
				♀♀ of *Heterodera*	formation of giant cells		(107)

while feeding, remain outside the host tissue except for the spear, against endoparasitic species that are entirely enclosed within the plant tissue while feeding; and (*b*) migratory species (which leave the parasitized tissue after a short feeding time to move to a new site), versus sedentary species (which remain at a chosen feeding site) (20).

One migratory species may be more sedentary than another (e.g., *Criconemoides* versus *Tylenchorhynchus*). Within a species, larvae and males may be migratory while the females are sedentary (e.g., in *Tylenchulus semipenetrans*) (25, 149). In Table I, the most important genera of nematodes attacking plant roots are grouped according to their parasitic habits.

In most species (the leaf and bud nematodes, the stem nematodes, and most of the root-infesting species), the egg is the only immobile stage. There is no special infective stage. In *Ditylenchus dipsaci*, invasion of plants is accomplished mostly by fourth-stage larvae, not because the other stages could not do the same, but because the fourth larval stage is the most numerous and best fitted to survive in moist soil or dry plant material (123). Migratory stages are practically always infective too. An infective stage may become sedentary shortly after penetration into plant tissue (second larval stage of *Heteroderidae*, young females of *Rotylenchulus*, *Tylenchulus*, *Nacobbus*), or may remain migratory within the plant (second-stage larvae of *Anguina tritici* and *A. agrostis*). Truly sedentary stages are saccate and the body musculature is so reduced that only the head can move (*Heteroderidae*, *Tylenchulidae*, *Nacobbus*, *Rotylenchulus*). *Hemicycliophora*, *Criconemoides*, and *Paratylenchus* species often feed for a long period in one place on a root, but from time to time they move to other places, therefore, they are not truly sedentary.

Host Plants and Population Dynamics of Nematodes

For the sake of simplicity, host lists divide the plant species into two groups: hosts and non-hosts. A plant is a host to a nematode species if the latter can derive sufficient food from the plant to sustain growth and multiplication. On a non-host, a nematode cannot complete its life cycle although some development may occur.

The presence of a nematode in a plant is not a sufficient reason for calling the latter a host although this has often been done. Many non-hosts are invaded, e.g., *Crotalaria* and *Tagetes erecta* by *Meloidogyne* sp. (5, 19), *Beta patellaris* by *Heterodera schachtii* (70). *Hoplolaimus uniformis* has been seen puncturing the roots of the non-host *Cucumis sativus* (131), and many more cases can be found in the literature. Nor is the mere existence of symptoms ordinarily associated with the presence of nematodes a certain indication that a plant is indeed a host to these nematodes. Thus, Quanjer (119) wrongly listed flax as a host of *Ditylenchus dipsaci* because it was stunted and distorted in the presence of this nematode. This reviewer has found that *D. dipsaci* does not enter this plant (128). A plant is not definitively a host unless the life cycle of a given nematode can be completed upon or within it.

In some cases this evidence can be derived from the stages of development found in or on a plant. Thus, the presence of females with eggs of *Heterodera* and *Meloidogyne* in a plant is such evidence, as only second-stage larvae invade roots.

Our knowledge concerning nematodes able to complete life cycles on certain plant species is still very meager, however. Host plants range from good hosts, which can support a large population, to poor ones upon which only a small population of a nematode can live. For devising crop rotations intended to keep the population density of a certain nematode species low, it is of little use to know whether or not a plant should be called a host, but very important to know how good a host it is. General information regarding plants as good or poor hosts to a given nematode is very often found in literature, but more precise data have been collected in relatively few cases, e.g., for several *Heterodera* species (33, 62, 73, 77, 161) *Belonolaimus gracilis* (65), and *Tylenchorhynchus claytoni* (82). The degree of excellence of a plant species as a host has been indicated in two ways: (*a*) by the rate of increase of a low initial population density in a specific time period [(65); the *f* index of Winslow (159); the cyst efficiency of Hesling (61)], and (*b*) by the maximum population density [ceiling level of Jones (73); potential increase ceiling of Duggan (33)], which a plant species can support under certain conditions.

The rate of increase of a nematode population on a host plant depends on the density of that population (15, 33, 54, 73, 74, 131, 135, 158). In some experiments with *H. schachtii* (44, 74) and with *H. rostochiensis* (158), all initial population densities increased or decreased to a "ceiling level" on good hosts. In others (15, 33, 54), the final population densities had a maximum at medium initial population densities. All experiments have in common the finding that the higher the initial population density the lower is the rate of increase. Little thought has been given to finding an explanation for this phenomenon. Relatively low final population densities resulting from high initial ones (15, 33) have been ascribed to damage inflicted upon the host plant. Considerable reduction of the root system by a heavy invasion may undoubtedly reduce the chance of survival of the attacking nematodes, but it is certainly not the only factor involved. Populations of *H. schachtii* reached a ceiling level on cabbage which was not seriously damaged (73). In experiments with *Hoplolaimus uniformis*, the roots of the plants were damaged very little even where initial and final population densities were the same (131). Moreover, the highest population levels that can be reached vary under different soil conditions (73, 131) and for different host plants or varieties of the same plant species (73, 158). It is much more likely that intraspecific competition rather than reduction of the available amount of food is the chief cause of the decrease of the rate of multiplication at increasing population densities. With increasing population density there is an increasing possibility that different animals will try to feed in the same place, although a more uniform distribution would provide abundant food for all (1, 106). Therefore, most probably a similar relationship between initial and

final population densities exists for poor hosts at low population densities as for good hosts at higher ones. Good hosts are characterized by a high "ceiling level" of the population density, poor hosts by a low one. At very low initial population densities, the rate of increase will approach a limit that does not necessarily bear a strong relation to the population "ceiling."

A full characterization of the host status of a plant in relation to a given nematode can be made only after investigating the rate of increase of this nematode at initial population densities ranging from very low to well above the ceiling level for the plant species under investigation. The results of such experiments will also be influenced by environmental conditions. There is a fairly extensive literature on the influence of ecological factors on the activity of nematodes (76), but no information on the influence of these factors on nematode population dynamics.

The host status of a plant in relation to a particular nematode species depends upon a number of factors: attraction of the nematode by the plant, inducement of the nematode to penetrate into the plant or to try to feed on it, the composition of the materials upon which the nematode tries to feed, and the reaction of the plant to the activities of the nematode.

Attraction of Nematodes by Host Tissue

A certain ability of the vagrant or infective stages of plant-parasitic nematodes to locate a tissue suitable for feeding would seem necessary for their survival. Linford (92, 93) was the first to demonstrate that larvae of *Meloidogyne* are attracted to the region of cell elongation of roots of host plants, and also to decomposing plant tissue and yeast colonies. Wieser (156) found that the apical 2 mm. of tomato roots were repellent, but the next 6 mm. were attractive to *Meloidogyne hapla* larvae. In sterile root cultures, Peacock (116) found tomato root tips to be strongly attractive to *Meloidogyne incognita* larvae, the appeal appearing to increase when the root was punctured. The larvae were attracted to the root tip even when separated from it by cellophane. There was no indication of repellence in Peacock's experiments. Wallace (153, 154) observed that larvae of *Heterodera schachtii*, *H. major*, and *H. rostochiensis* were attracted by seedlings of host plants, but Bergman & van Duuren (8) could not confirm this for *H. schachtii* and beet seedlings under sterile conditions. In their experiments the larvae moved at random. These authors suggest that microörganisms in the root zone were the true source of attraction in Wallace's experiments.

Larvae of *H. schachtii* and *H. rostochiensis* were attracted very little or not at all by root diffusates in the absence of roots (8, 87, 155). The attraction of *Pratylenchus pratensis* (=*coffeae*?) and of *Meloidogyne* sp. to host roots depends upon the rate of growth of the latter (42).

According to Bird (9), the attractiveness for *Meloidogyne javanica* larvae of organic substances occurring in root exudates of tomato is associated with their negative redox potential, but later (10) he doubts whether this is the only important factor in the attraction of nematodes to host roots.

Oxygen was not effective as an attractant to *Meloidogyne* larvae. Klingler (78) has stated that stem nematodes congregated in areas having a slightly higher CO_2 content than the surrounding medium. When brought into a redox potential gradient they first moved in the direction of the potential but reversed their direction long before the redox potential gradient had disappeared. In other experiments, stem nematodes were also attracted by substances with a positive redox potential difference (79). It is not very probable, however, that stem nematodes are guided to stem parts of host plants by CO_2 gradients only. The many CO_2 sources in the soil would distract them too much, and the result would be little more than a random search. Plants can be good hosts to certain *Heterodera* species only if their roots produce a substance which induces quiescent larvae of these species to hatch from eggs. In summation, it can be said that the mechanism of host finding by plant-parasitic nematodes is still little understood.

Penetration of Nematodes Into Host Tissue and Feeding

In above-ground areas nematodes may enter through the stomata (*Aphelenchoides* species) and travel through intercellular spaces. Roots cannot be invaded, however, without destruction of cell walls of epidermal and often of cortical cells. The nematode first weakens the cuticle or other cell walls by a number of jabs with the stylet in a small area, after which the head is pushed firmly against the weakened spot until the cell wall ruptures (80). *Ditylenchus dipsaci* was seen to enter stems through stomata and through openings in the epidermis, which were produced presumably by the action of saliva excreted by the nematode while still outside the plant (119).

There are virtually no data available to explain the forces that induce nematodes to puncture cells and to try to feed on them. Dickinson (29) has found that larvae of *Heterodera schachtii* attach themselves more easily with their lips to hydrophobic nitrocellulose membranes than to hydrophilic ones. Only the former also were punctured with the stylet. Dickinson concludes that adhesion and attempted penetration are responses to a purely physical stimulus, and that root hairs would not be suitable for nematode feeding as they have hydrophilic cell walls. The latter conclusion is in contradiction with observations made on the feeding of *Tylenchorhynchus dubius* which often punctures root hairs (80), as does *Hoplolaimus uniformis*, occasionally.

In the feeding of larvae and adult females of *Meloidogyne* sp. Linford (90, 91) could distinguish two phases: (*a*) puncturing of the cell wall with the stylet and extrusion of digestive secretions into the cell through the stylet and (*b*) withdrawal of perhaps predigested materials from the cell. These two phases are also quite separate in *Tylenchorhynchus dubius*, where each requires less than half a minute (80). Following this short period of feeding, the animal moves away to try its luck in another section. In contrast to this behavior, *Hemicycliophora* may feed for hours and even days on the same cell. In the latter case, the introduction of saliva and withdrawal of cell contents are not separate actions. The nematode sucks part of the cell

contents into its esophagus by expanding the lumen of the median esophageal bulb; at the same time, the volume of the punctured cell decreases. However, when the lumen of the esophageal bulb returns to normal, the cell expands again. Obviously, some of the contents flows back from the esophagus of the nematode into the parasitized cell. As saliva is produced simultaneously and continuously by the nematode, some of this material also flows into the cell (131).

Reactions of Invaded Tissue of Host Plants

Most root-infecting nematode species and some of those infecting aboveground parts of their host plants, can withdraw food from both undifferentiated and differentiated cells, probably after a period of intracellular predigestion by injected saliva (see Table I). Distortion of the nucleus (80, 83) and discoloration of the cell wall and of the cell contents, which indicate that the punctured cell is dying or is already dead, may be the result of the activities of these nematodes. Root hairs and epidermal cells upon which *Tylenchorhynchus claytoni* or *T. dubius* have fed, show no reaction at all (80, 83). The death of cells is, of course, of no advantage whatever to the plant-parasitic nematodes living on them. Plants which react with little necrosis to invasion of a nematode species can maintain larger populations than those showing extensive necrosis. [*Pratylenchus zeae* on tobacco varieties Hicks and Bottom Special versus Dixie Bright 101 (100), and on maize versus tobacco (52); *Radopholus similis* in sterile versus non-sterile citrus roots (32); *Pratylenchus penetrans* in rye, oats, and barley versus roses and apples (108).]

The inhibition of mitosis in apical meristems and the swelling of root tips caused by *Trichodorus*, *Belonolaimus*, *Hemicycliophora*, and some other species, the swellings of roots caused by *Meloidogyne* sp., and the stunting and distortion of stems caused by stem nematodes (*Ditylenchus dipsaci*) are of no direct importance to the feeding of these nematodes. They also occur in some poor and non-hosts [*Ditylenchus dipsaci* (128, 129); *Meloidogyne* sp. (115)], and are absent in some good hosts or parts of hosts having tissue that is very suitable for feeding [*D. dipsaci* in potato tubers (119); *Meloidogyne* sp. on cereals]. The stunting of stems by *Ditylenchus dipsaci* is associated with the development of lateral buds and this may result in providing more stems, i.e., more food for the increasing nematode populatiln. Undoubtedly, swollen roots provide more room for developing root knot nematodes than do normal ones. Therefore, these symptoms of attack may indicate a more complicated relationship between parasite and host than is found in most root-infesting nematodes.

The so-called "giant cells" are essential to the feeding and development of *Heterodera* and *Meloidogyne* spp. in roots, however. Infective (second-stage) larvae of the nematode species penetrate into the root tissue just behind the root cap and take up a longitudinal position in the periblem with the head in the plerome (later in the pericycle) (7, 17, 105). Probably as a result of

injection of saliva by the nematode, the plerome cells near the head of the nematode do not develop into normal vascular tissue. They enlarge, instead, and their nuclei divide without cell walls being laid down. Walls between cells may even break down and several cells may coalesce giving rise to "giant cells." These are densely packed with granular protoplasm and may extend into both the stele and the cortex of the root (17, 81, 107). In potato roots even the larvae of *Heterodera rostochiensis* which lie entirely within the periblem, may induce giant cell formation in the adjacent plerome, while the periblem cells surrounding the larvae show little reaction at first (98). The giant cells are comparable to the feeding cells lining the cavities of galls of cynipid wasps.

According to one writer's findings, it appears that giant cells are not always necessary for the feeding of *Meloidogyne* sp. Christie (19, 21) reports two cases of *Meloidogyne* feeding and life cycle completion on unchanged cells: on callus tissue of the non-host *Pelargonium graveolens* and on tubers of *Caladium* sp. He also mentions a case of *Meloidogyne* developing in the root nodules of a resistant soybean variety. His explanation of these phenomena is that in the mentioned plants the roots are resistant because the nematode cannot prevent the cells from becoming differentiated. However, tissues remaining undifferentiated can supply the nematode with sufficient food without any alteration. Galls with a structure resembling that of galls of cynipid wasps are formed on roots of certain grasses by *Ditylenchus radicicola*, (47) on stems and leaves of mosses, *Gramineae* and a few dicotyledons, and in the flowers of several *Gramineae*, *Primula*, and *Amsinckia* by several *Ditylenchus* and *Anguina* species. The nematodes live in a cavity in the gall lined with thin-walled feeding cells (48).

In *Anguina tritici*, *A. agrostis*, and *A. klehbani* the highest degree of parasitic specialization and adaptation of plant-parasitic nematodes to the life cycle of the plant is found. This is associated with low numbers of host plants per nematode species, as opposed to the large host ranges of the root-infesting nematodes with a primitive type of parasitism. In *Anguina tritici* and *A. agrostis*, second-stage larvae coming from old galls in the soil infest young seedlings of their host plants, wheat, and *Agrostis stolonifera*, respectively. They live ectoparasitically between the young leaves until the embryonic inflorescence has developed, whereupon they enter the floret primordia and quickly develop into adults, a few only in each flower. By the time the seed ripens, the attacked ovaries have changed into galls containing thousands of coiled, dry, second-stage larvae. When galls, mixed with the seed, are sown the life cycle can be repeated.

Ditylenchus dipsaci, the stem and bulb nematode, has an intermediate position between the ecto- and endoparasitic *Ditylenchus* species which simply puncture cells for feeding (*D. destructor*, *D. drepanocercus*, *D. myceliophagus*), and the gall-forming species (*D. radicicola*, *D. askenasyi*, *D. graminophila*, for example). For the feeding and multiplication of *D. dipsaci*,

it seems to be of vital importance that the middle lamellae in the infested tissue be dissolved by the saliva of the nematode. The cells, which are often completely loosened from each other, do not undergo further changes apart from distortion of their nuclei (83) and ultimate necrosis. The swelling and distortion of the affected part of the plant is attributable partly to cell proliferation which makes available for the nematode an increased amount of feeding tissue. Such distortion might be viewed as a very incomplete effort toward gall production.

The occurrence of altered cells upon which the nematodes live is always associated with a sedentary mode of life of the nematode. However, it seems that not all sedentary nematodes live in galls or on special cells. Whether or not changes similar to those caused by *Heterodera* and *Meloidogyne* are associated with the feeding of the sedentary stages of *Nacobbus, Rotylenchulus*, and the *Tylenchulidae* has been investigated only superficially. Peacock (114) reports slight necrosis as the only visible effect of *Rotylenchulus* on host roots.

Enzymes in Nematodes

The changes in host tissues described above are thought to be caused by the nematode saliva or other excretions. Homogenates of whole *Ditylenchus dipsaci* and *D. destructor* contain cellulase and chitinase, but pectinase was found only in one of several homogenates (146). Homogenates of *D. triformis, D. dipsaci*, and *Pratylenchus zeae* contain enzymes that are normally associated with the digestion of food in animals (84). Sterile *Pratylenchus penetrans* probably produce emulsin. This hydrolyzes amygdalin present in sterile peach roots and in agar. The resulting HCN is responsible for the killing of root tissue, thus causing the extensive lesions that are characteristic of attack by *P. penetrans* in roots of peach (104).

Reactions of Poor Hosts (Resistant Plants) to Invasion

The efforts of a nematode to feed on a plant may not be very successful for different reasons. Krusberg (83) obtained multiplication of *Pratylenchus zeae* on callus tissue of the poor host, lucerne, by adding coconut milk to the medium upon which the callus was cultured. Apparently, the lucerne is a poor host because it lacks some substance or substances that are present in the coconut milk.

Little reaction and no giant cell formation in roots invaded by *Meloidogyne* and associated with failure of the nematodes to develop, was observed in *Tagetes erecta* (19, 21), and is undoubtedly a wide-spread cause of resistance of plants to these nematodes. Rye and oat plants resistant to *Ditylenchus dipsaci* develop, in part, normal symptoms of attack which, however, remain restricted to very small areas of the plant tissue. The stem nematodes found in these areas develop very slowly or not at all (13, 129). Red clover plants may be resistant to the red clover race of *D. dipsaci* for the same reason (35).

Necrosis as a reaction of resistant plants to invasion by *Meloidogyne* sp. was described by Barrons (5) for lima beans and *Crotalaria*, and by Chitwood; Specht & Havis (16) for peach. It becomes apparent within a few days after the *Meloidogyne* larvae penetrate into the roots and can therefore be used in breeding programs as a quick indication that a plant is a poor host (28). Necrosis of invaded tissue is associated with resistance against stem nematodes in many plants (50, 128), in *Beta patellaris* against *Heterodera schachtii* (7, 69), and in certain lines of *Solanum tuberosum* subsp. *andigenum* and crosses with *S. tuberosum* against *H. rostochiensis* (86). In the latter cases, the cells which, in susceptible plants would form "giant cells," become necrotic. The reaction is not the same in the entire root system, however. *Heterodera schachtii* larvae show some development in certain resistant *Beta* species (136); the same is true of *H. rostochiensis* on *Solanum nigrum* (31).

In potatoes resistant to *H. rostochiensis*, very few females but a relatively high number of males reach the adult stage (68, 157). In these poor hosts, a limited number of the invading nematodes may be able to withdraw some food from cells which do not become necrotic immediately or which even develop into giant cells to some extent. Differences between susceptible and resistant plants must be small as plants that are resistant to one race of *H. rostochiensis*, for example, or *Ditylenchus dipsaci*, may be susceptible to another race of these nematodes.

"Enemy Plants"

Some plant species possess a combination of characteristics which may be very detrimental to populations of specific nematodes. *Tagetes erecta* and some *Crotalaria* species are heavily invaded by larvae of certain *Meloidogyne* species. The latter fail to develop extensively in these roots but, nevertheless, cannot leave them and die within about 8 days (21, 53). Had these larvae remained in the soil, they might have lived much longer. Whether the reduction of populations of *Pratylenchus penetrans* and *P. pratensis* in soil where *Tagetes* is grown (109, 138), is caused in the same way is not known.

A number of poor hosts or non-hosts of *Heterodera rostochiensis* and *H. schachtii* induce quiescent larvae to hatch from eggs. Whether or not these larvae enter the roots of these plants they generally die fairly soon after hatching (see below).

Hatching of Nematode Eggs Through the Influence of Materials Diffusing From Plant Roots

The eggs of most species of plant-parasitic nematodes seem to hatch without regard for the presence or absence of host tissue in their immediate vicinity. Eggs of *Ditylenchus dipsaci* (11, 123) and of *Tylenchulus semipenetrans* (149) were found to hatch very well in pure water. Hatching of eggs of *D. radicicola* was not increased in water which had been in contact with host roots (88). However, eggs of the majority, though certainly not all *Heterodera*

species, hatch much more readily in water which has been in contact with host roots. This phenomenon was first detected with *Heterodera schachtii* (6) but is most pronounced with *H. rostochiensis*. Only a small percentage of the eggs of this species hatch in water (147). The same applies to *H. cruciferae*, *H. carotae*, *H. galeopsidis*, *H. humuli*, and *H. trifolii* (160). A considerable percentage of the eggs of *H. schachtii* may hatch in water, however, although a much higher percentage hatches in root diffusates of host plants (110, 160). Some of the hosts of *H. cruciferae* do not produce a hatching stimulus for this nematode, although they do so for eggs of *H. schachtii* to which they are hosts as well (160). There is no stimulation for the hatching of eggs by any host root diffusate in *H. avenae* (59, 126, 160) nor in *H. goettingiana* (43), although there may be some stimulation of these species and of *H. glycines* by growing pea roots (40, 70, 160). The tendency of larvae of some *Heterodera* species to remain quiescent in the eggs until host roots are within reach of the hatched larvae greatly increases the chance of survival of the nematode. Such behavior has been considered an expression of the degree of parasitic specialization of the nematode (76, 160). Indeed, it is associated with low numbers of host species in certain *Heterodera* species, but *H. glycines*, the eggs of which hatch without an external stimulus, survives and spreads equally well on a low number of host plants. Expressed in a percentage hatch, the response of eggs of *H. rostochiensis* to a hatching stimulus of constant concentration is normally distributed against the logarithm of time (36, 37). The percentage of the exposed eggs that hatch is proportional to the logarithm of the concentration of the hatching agent at suboptimal concentrations of the latter (38). Plants frequently show a high degree of coincidence between stimulating the hatching of eggs of a certain *Heterodera* species and being hosts to the species. Such coincidence suggests that the production of a hatching stimulus is linked to one or more factors in the plant that are essential to the feeding and development of the *Heterodera* species to which it is host. However, certain plant species produce a hatching stimulus without being hosts (so-called "enemy plants") (68, 69, 111, 124, 157, 160). These plant species are, for the most part, closely related to host species. They are often invaded by the stimulated larvae which, in some of the mentioned plant species, develop in some degree to males but only very rarely to females (68, 136, 157). As *Heterodera* larvae cannot live in the soil very long (113, 137), the possibility of control of *H. schachtii*, with the help of "enemy plants," has been investigated. However, the reduction of populations of this nematode under *Hesperis matronalis*, the most promising "enemy plant," did not exceed in any practical degree the reduction under an immune crop (112). On the contrary, growing potato varieties resistant to *H. rostochiensis* results in a much greater reduction of high populations of this nematode than that achieved by growing immune crops (26, 45, 68, 158). Although eggs of *Meloidogyne* species hatch rather well in water, hatching was somewhat higher in diffusates from tomato roots (152). Eggs of *Heterodera*

rostochiensis in soil are not stimulated to hatch when further removed than 2 cm. from host roots (155).

A considerable amount of research has been carried out to isolate and determine the chemical composition of the hatching factor produced by potatoes and tomatoes, with the ultimate aim of using it for controlling *H. rostochiensis* (12, 71). So far, this research has been only moderately successful, and it is doubtful whether the natural hatching agent can ever be used for the treatment of infested soil as it breaks down rapidly (39).

Disease Complexes in Which Nematodes Play an Important Part

Most experiments reported on the effect of a certain nematode species on a certain plant were not done under aseptic conditions. Under the best controlled experiments, plants growing in the presence of an undefined number of bacteria and fungi were compared with plants growing under identical conditions but for the presence of a specified nematode species. Whether or not a nematode is an important factor in the etiology of certain pathological changes of plant tissue or reductions in yield can be established very well by such experiments but the question of participation in the syndrome by other organisms will remain unanswered. Inoculation of aseptic plants with aseptic *Meloidogyne* sp. (116), *Ditylenchus dipsaci* (83), gave no results differing from those under more natural conditions. Root swelling, giant cells, necrosis after heavy invasion, dissolution of middle lamellae is exclusively ascribable to the action of the nematodes. Also, under aseptic conditions root lesion nematodes (*Pratylenchus* spp.) can cause severe necrosis in host roots (101, 102, 103, 138).

There are a number of cases, however, in which the pathological condition of plants attacked by nematodes is influenced by secondary invaders. They can be divided into three groups: (*a*) The nematode is the chief incitant of the pathological changes of the plant tissue which, however, are aggravated by non-specific secondary invaders as in attacks by *Pratylenchus penetrans* (56, 57, 104, 138); (*b*) there is much difference in symptoms caused by the nematode alone and when associated with soil organisms. Aseptic citrus roots attacked by aseptic *Radopholus similis* form tumors by cell division in the pericycle. In non-sterile roots such cell proliferations do not develop to a great extent, but a cortical necrosis resulting in large cavities in the root occurs instead. The sterile roots can support very large populations of the nematode which, however, leave the root when it becomes infected. Therefore, non-sterile citrus plants contain much smaller populations of the burrowing nematode than do sterile plants and are less seriously damaged (32). This is a clear warning not to assume without proof, as has been done too often, that secondary invaders aggravate a nematode attack or are the main cause of damage done to the plant. (*c*) Certain diseases caused by organisms may gain entry into the plant only with the help of nematodes. Attack by the nematode alone may inflict another type of damage on

the plant. The classical example of such interaction between a nematode and another organism is cotton, resistant to *Fusarium* wilt but susceptible after attack by *Meloidogyne incognita*. Observations indicating that this relationship exists were made as early as 1892 (2) and appeared repeatedly thereafter, but pertinent proof was not produced until 1956 (97). Attack by *Belonolaimus gracilis* also strongly increases the susceptibility of cotton to wilt (66). A similar case is "early yellowing" of peas caused by a combined attack by *Fusarium oxysporum* forma *pisi* race 3 and *Hoplolaimus uniformis* (89). *H. uniformis* alone scarcely damages pea roots. Cauliflower disease of strawberries is caused by *Corynebacterium fascians* but this organism will not enter the plant without the help of *Aphelenchoides ritzemabosi* which, alone, causes alaminate leaves and proliferation of secondary crowns (27, 118).

In none of these cases could the role of the nematodes be replaced by mechanical damage to the test plants. The incidence of bacterial wilt in carnations and of Granville wilt in tobacco is increased both by nematode attack and by mechanical injury (4, 96, 142). In these cases, nematodes are not essential to the occurrence of the disease. A few nematode species transmit soil-borne viruses: *Xiphinema index* (63), *X. diversicaudatum* (55), and *Trichodorus pachydermis* (139).

Damage Caused by Nematodes to Plants

The economic damage caused by plant-parasitic nematodes is the chief reason for the extensive research that is done on these animals in most parts of the world. Such causation might imply that methods have been developed and applied that can provide reliable information on the magnitude of the damage. Unfortunately, this is true only to a limited extent. The amount of destruction that can be expected from a certain nematode species can be determined only by measuring yields of crops grown in the presence of different numbers of this nematode, while further growing conditions remain the same throughout the experiment. This can be done in pot experiments but is by no means easy in field trials. The crop loss caused by some of the "classical" plant-parasitic nematodes (*Ditylenchus dipsaci, Heterodera schachtii, H. rostochiensis, Meloidogyne* sp.) is so large that it far outweighs differences in fertility. Moreover, the presence of these nematodes is easily recognized by the occurrence of specific symptoms or of cysts. Very often, damage inflicted by *Pratylenchus* species and other migratory nematodes is by no means clear cut, however. Populations of these nematodes tend to be relatively low throughout whole areas and, consequently, damage is slight if present at all. In many cases, the difference in yield between untreated plots and plots receiving a nematocide has been attributed entirely to nematode attack. This, of course, is a highly unreliable way of measuring the damage done by nematodes (132).

As the rate of multiplication of nematodes on plants is always relatively

low compared to that of certain insects and fungi, the population density before sowing or planting a susceptible annual crop largely determines the amount of damage that will be done to this crop. The results of all experiments made with a sufficient range of initial population densities agree in that there is a more or less linear regression between the yield and the logarithm of the population density of the nematode (60, 73, 95, 132).

For different nematodes, the destruction begins at different population levels. Very small numbers of *Ditylenchus dipsaci* may damage onions, rye, carrots, and other plants extensively (127, 132). Populations of one to five nematodes per 500 gm. of soil of the species *Pratylenchus penetrans* may produce far-reaching losses among daffodils (138), whereas damage by this nematode in potatoes becomes apparent only at more than 500 nematodes per 500 gm. of soil (108). Injury to beets by *Heterodera schachtii* begins at 2000 to 5000 larvae per 500 gm. of soil (59, 73). There are some reports in the literature on an increase of yield caused by a light attack by nematodes (74, 95). In case of attack by root-infesting nematodes, the root weight of certain plants may be reduced by much lower populations than the top weight would be (99). Obviously, such plants make larger root systems than is necessary in order to give good top growth. Certain varieties of rice are so well adapted to invasion by *Radopholus oryzae* that in the absence of this nematode they will tiller excessively, thereby reducing the yield considerably (151).

Damage and Host Status of the Damaged Plant

There is no close correlation between the host status of a plant relative to a nematode species and its susceptibility to damage by the nematode. Potatoes resistant to *Heterodera rostochiensis* are about as seriously injured by this nematode as are susceptible potatoes (158). The peach varieties Shalil and Yunnan, which are resistant to *Meloidogyne incognita*, are as severely damaged when planted in soil that is heavily infested with this nematode, as are susceptible varieties. Certain tobacco varieties, known to be good hosts to *Pratylenchus zeae*, sustain much less tissue injury from this nematode than do other varieties which support smaller populations (100). As nematodes multiply relatively slowly, it is the reaction following invasion which causes most of the damage. Heavy necrosis following invasion in a poor host may be as severe as the formation of giant cells in a good host. The amount of tissue loss depends upon how tolerant is the invaded tissue to the invading nematode. Therefore, it may be of little use to grow a resistant but intolerant plant (one that suffers much destruction) in a heavily infested field, for example, resistant potatoes in a field infested with *Heterodera rostochiensis*. Resistant but intolerant varieties can be used in situations in which the nematode population is first built up on the damaged crop (stem nematodes on red clover or lucerne). If dangerous population densities are built up on other crops, tolerant varieties, whether resistant or not,

must be chosen. On tolerant but non-resistant plants, higher populations of nematodes may multiply in so brief a period that tissue injury will equal that sustained by intolerant varieties. Therefore, each local situation will determine what selection should be made, tolerant but susceptible or tolerant and resistant. Mountain (103) attempted to measure the degree of tolerance of a number of tobacco varieties to *P. penetrans* and *P. minyus* and to express it in terms of the population density of those nematodes which caused 50 per cent reduction of the weight of the test plant. The tolerance of tobacco appeared to depend upon temperature. For *P. penetrans*, tolerance was lower at low temperatures. for *P. minyus* it was lower at high temperatures.

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SUGAR TRANSPORT IN MICROORGANISMS[1,2]

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For the general physiologist, almost all processes begin at the cell surface. Osmoregulation, excitability, muscle contraction, propagation of the nerve impulse, intestinal absorption, secretory activity, and the origin and maintenance of bioelectric potentials are intimately associated with the cell membrane. The microbial physiologist, by contrast, has a different orientation: dealing with organisms of limited size, with tremendous reproductive potential and intense biochemical activity, he is concerned mainly with growth and nutrition and a variety of biochemical phenomena, e.g., enzymatic constitution, metabolic pathways, and protein and nucleic acid synthesis. Microbial permeability and mechanisms of membrane transport phenomena have, until recently, been neglected or methodologically eliminated by the ball mill, the Waring blender, or the sonic oscillator. As a consequence, many questions about the cell membrane have accumulated, and the explanations of what it does in the microbial cell are essentially speculative.

Some of these questions are derived from nutritional studies (which will also serve to introduce the importance of membrane transport phenomena emerging from other kinds of information). A problem which has always plagued nutritionists is how to interpret the inability of an organism to utilize certain kinds of compounds: is this due to an internal enzymatic deficiency or is the cell impermeable to the non-utilized compound?

In the absence of other data, inability of a cell to use one of several sugars or the acids of the tricarboxylic acid cycle, for example, could reflect a lack of appropriate kinases or lack of a Krebs cycle, or a lack of a transport mechanism for uptake of the individual substrates. For many microorganisms both explanations have been offered. Cell-free preparations are often used to distinguish between these two alternatives. If the method of preparation has not destroyed the enzyme, the absence of activity in disrupted cells usually denotes an enzymatic deficiency. A demonstration that cell-free preparations can metabolize a compound not used by intact cells is usually taken to mean that the cell membrane is impermeable to that particular compound.

This question of permeability arises whenever there is a discrepancy between the enzymatic capacity of cell-free preparations and the activity of the intact cell. Doudoroff *et al.* (1) illustrated this situation in a mutant of *E. coli* which could not utilize glucose although it metabolized maltose and con-

[1] The survey of the literature pertaining to this review was concluded in December, 1960.

[2] The following abbreviations are used: ADP (adenosine diphosphate); ATP (adenosine triphosphate); DNP (dinitrophenol); FDP (fructose diphosphate); α-MG (methyl-α-D-glucoside); PABA (p-aminobenzoic acid).

tained hexokinase. This was the first of many examples of what has since been called "crypticity."

Barrett *et al.* (2) and Kogut & Podoski (3) then focussed attention on the cell membrane in adaptation phenomena when they showed that an adaptation was necessary for whole cells to oxidize Krebs cycle acids, while the cell-free preparations of both adapted and non-adapted cells were equally able to oxidize these substrates. The recent literature contains many examples of crypticity and adaptations associated with the induction of membrane-transport systems. As an excellent example, Eagon & Williams (4) described cryptic phenomena associated with sugar utilization by *Pseudomonas aeruginosa*. In this case (*a*) glucose-grown cells fermented only glucose, but cell-free extracts phosphorylated fructose as well since both hexokinase and fructokinase were present; (*b*) fructose-grown cells fermented only glucose and fructose; however cell-free extracts phosphorylated glucose, fructose, and mannose, since growth in fructose resulted in the appearance of mannoisomerase which can isomerize mannose to fructose; (*c*) only mannose-grown cells fermented all three sugars. Fructose should have been fermented by glucose-grown cells, and mannose should have been fermented by fructose-grown cells had the sugars penetrated the cell membrane. Growth in the individual sugar seems required for induction of specific transport mechanisms.

Once it was recognized that utilization of certain substrates is controlled by transport mechanisms, it was realized that certain inhibitors of growth or metabolism could affect membrane transport rather than an inhibition of later metabolic reactions. Durham & Hubbard (5) for example, showed that *p*-aminosalicylic acid inhibited *p*-aminobenzoic acid oxidation in *Pseudomonas fluorescens* by blocking PABA uptake. Uranyl ion has long been known as a specific, non-penetrating inhibitor of sugar metabolism; it inhibits sugar transport in yeast [Rothstein (6); Cirillo (7)]. It also prevents sugar uptake by *Neurospora* [Cochrane & Tull (8)]. Probably the same possibility holds for inhibition of certain adaptation processes which result in "diauxie." Thus, glucose inhibition of galactose adaptation by bakers' yeast has been attributed to inhibition of galactose transport [Burger *et al.* (9)].

Nevertheless, the demonstration that a differential effect depends on the presence of an intact cell membrane, while suggesting some participation of the cell membrane, cannot be taken as proof that a transport system is involved. In the absence of a direct and specific demonstration of an effect on the hypothetical transport phenomenon, proposals of permeability mechanisms to explain such data merely swell the literature with more instances in which "permeability" is used as a synonym for ignorance.

The purpose here is to review literature in which the claim for a special role of the cell membrane in transport phenomena has been demonstrated. This review is largely devoted to papers concerned with sugar utilization and which do illustrate a specific transport phenomenon or else advance understanding of the mechanism of transport phenomena.

Classification of Transport Phenomena

Two types of transport mechanisms are recognized: active and passive. Passive transport is further classified as (*a*) simple diffusion, and (*b*) facilitated diffusion. Classification of transport phenomena as passive or active usually rests on two criteria: dependence upon metabolic energy or movement of a substance against its electrochemical difference (i.e., uphill transport).

Active transport.—If a transport process requires metabolic energy, it is called "active"; if an energy-dependent transport results in accumulation against a concentration difference,[3] transport is definitely active. [According to Rosenberg (10), movement of a substance against a concentration difference is the only certain criterion of active transport.]

Besides metabolic dependence and uphill transport, active transport usually shows the following additional characteristics: [Wilbrandt (11); Crane (12)], (*a*) rate of transport approaching saturation with increasing external concentration (variously described as obeying a Langmuir adsorption isotherm, or obeying Henri-Michaelis or Michaelis-Menten kinetics); (*b*) the rate of transport is specific for isomers; (*c*) structurally similar analogues show competitive inhibition; (*d*) the transport is specifically sensitive to certain enzyme inhibitors. In microbial systems, numerous sugars, sugar derivatives, amino acids, organic acids, purines, and some growth factors are transported by active transport (*vide infra*).

Simple diffusion.—If transport is not metabolically dependent or results in accumulation against a concentration difference, it is usually called diffusion. In the classical concept of membrane transport by simple diffusion formulated by Overton (13), Ruhland & Hoffmann (14), and Collander & Bärlund (15), the rate of transport of a large number of low molecular weight compounds obeyed Fich's laws of diffusion. Unlike active transport, the energy for transport is derived from thermal agitation. For substances transported by simple diffusion, the rate of transport is a strict function of the concentration difference across the cell membrane, molecular size, and lipid solubility. [For a discussion of simple diffusion and its application to permeability across biological membranes, consult Hober (16), Davson (17), and Davson & Danielli (18).]

Facilitated diffusion.—While simple diffusion is a passive transport, all passive transport is not accomplished merely by simple diffusion. Many compounds, especially low-molecular, hydrophilic substances, like sugars, although transported passively (i.e., no energy is involved besides thermal agitation), do not enter by simple diffusion. The outstanding characteristics of such transport, seen largely from studies of sugar transport in primate erythrocytes, [Le Fevre (19); Widdas (20); Wilbrandt (11, 21, 22); Bowyer

[3] In accordance with the recommendation made by Crane (12), the term "concentration difference" will be used in preference to "concentration gradient."

(23)] are (*a*) rate of transport approaches saturation with increasing external concentration; (*b*) rate of transport is specific for stereo and optical isomers; (*c*) structurally similar compounds may act as competitive inhibitors; (*d*) transport is specifically sensitive to certain inhibitors; (*e*) transport shows rates greater than predicted from molecular size and lipid solubility; (*f*) the Q_{10} is frequently anomalous (usually about 3.0); (*g*) the transport process is often species- or strain-specific. The term, "facilitated diffusion" is given this sort of passive transport to distinguish it from simple diffusion [Danielli (24)].

The similarity between facilitated transport and active transport is striking. But distinguishing between facilitated diffusion and active transport is not so easy when the transported substance is a metabolizable substrate. This group of features shared by facilitated transport and active transport led Widdas (20) to suggest that active transport may be a two-step process: an energy-independent, facilitated diffusion (which would account for the non-linear kinetics, stereospecificity, competitive inhibition, and sensitivity to certain inhibitors) followed by an energy-dependent accumulation against a concentration difference. Recent experiments with yeast (7, 9, 25, 26, 27) have shown that sugar transport results from a facilitated diffusion resembling that seen in erythrocytes and some of the transport systems described for bacteria as "permeases" may also represent induced facilitated diffusion mechanisms.

Methods Used in Transport Studies

Transport of a compound by whole cells has been measured in two ways: (*a*) by its disappearance from the external medium; (*b*) by analyzing cell extracts. One way to follow the penetration of non-metabolized substances is by the volume-distribution technique [Conway & Downey (28)] in which approximately equal volumes of packed cells and medium containing the test substance are mixed. At intervals, aliquots are removed and the concentration of the test substance in the supernatant estimated. Correction for dilution by the extracellular medium is made by using a non-penetrating substance as a standard, such as dextran, inulin, Cl^- or $SO_4^=$, depending on the test system. From the calculated distribution volume, the penetration "space" can be determined, assuming that the penetrating material is neither concentrated nor diluted. This method has two disadvantages: (*a*) relatively large volumes of cells are usually needed, (*b*) the method is rather imprecise, e.g., if 100 ml. of yeast (which contains 80 per cent water by volume) is mixed with 100 ml. of a 2 per cent solution of substance X, a dilution to 1.1 per cent represents 100 per cent entry into the whole water space. However, since 33 per cent of the yeast is extracellular water (Conway & Downey) a dilution to 1.5 per cent results if no penetration of the cell occurs. Therefore, a difference in final concentration between 1.1 and 1.5 per cent represents the range from 100 per cent to no penetration. A dilution to 1.5 per cent versus 1.35 per cent represents no entry and 34 per cent entry, respectively.

Direct analysis for the penetrating substance in the cells is to be preferred and may be carried out with either washed or unwashed suspensions. In the latter case, correction for the amount of material trapped in the intercellular space is made by using a non-penetrating material such as those mentioned in the volume of distribution procedure. Intracellular material is usually released by boiling the cell suspension (5 to 20 min.) in water, or extracting with alcohol, trichloroacetic acid (TCA), or any other procedure that destroys the cell membrane. In using radioactively labeled substances, samples of cell suspension are usually collected and washed directly on Millipore filters, the filter transferred to a planchet, dried, and counted. The latter methods are described fully by Roberts *et al.* (29).

A common source of error in both the volume-of-distribution method and direct cellular analysis is caused by adsorption, particularly by the cell wall, sometimes amounting to more than 80 per cent of the total uptake [Britt & Gerhardt (30, 31)]. Corrections for adsorption are usually made by subtracting the uptake which occurs at 4°C. after very short exposure periods.

Bacterial and yeast protoplasts have recently come into more use in transport studies as they offer the following advantages over whole cells: (*a*) adsorption by the cell wall is eliminated (30, 31); (*b*) removal of the transported substance from the cell can be achieved by protoplast lysis (a far gentler procedure than can be used with whole cells); (*c*) the rate of transport can be estimated from photometric determination of volume changes [Mitchell & Moyle (32, 33)]. The latter procedure offers more than a methodological advantage since volume changes and lysis indicate also that the transported material is osmotically active in the cell, not bound to cell constituents [Sistrom (34); Wachsman & Storck (35)]. Except for the elimination of cell wall adsorption, spheroplasts offer the same advantages [the continued presence of cell walls in spheroplasts has been described by Gebicki & James (36)].

Development of techniques for preparing protoplasts of yeasts [Eddy & Williamson (37)] and molds [Bachman & Bonner (38)] and the possible applications to algae of recent techniques for preparing protoplasts from higher plants [Cocking (39)], should increase the use of protoplasts in transport studies.

Sugar Transport

From studies of sugar utilization by bakers' yeast, Rothstein (6, 40) proposed that the yeast cell membrane is impermeable to free sugars, and that utilization of metabolizable sugars depends upon phosphorylation at the cell membrane. This conclusion was derived from the following observations: (*a*) the yeast cell membrane was impermeable to non-metabolizable sugars (6, 28, 40); (*b*) the rate of sugar uptake obeyed saturation kinetics; (*c*) the rate, and to a certain extent the pathway, of sugar metabolism was influenced by changes in or additions to the external medium which did not alter the intracellular environment of the cell. (The most convincing evi-

dence was the fact that uranyl ion was a specific inhibitor of sugar uptake although it did not penetrate the cell membrane); (*d*) no free intracellular sugar accumulates during fermentation; (*e*) the yeast cell membrane is impermeable to inorganic phosphate; (*f*) inorganic phosphate is absorbed during sugar fermentation.

These observations were taken to mean that sugar utilization requires phosphorylation at the cell surface by hexokinase. However, these same data could be the result of a stereospecific, rate-limiting, transport step preceding phosphorylation. If so, substrate specificity, the saturation kinetics, and the effect of a non-penetrating inhibitor would be on the transport system, not a surface hexokinase.

That sugar utilization in yeast involves a step preceding phosphorylation has been suggested by several authors. Derrick *et al.* (41) reported that a yeast hexokinase antiserum inhibited sugar fermentation only in cell-free extracts while yeast phosphatase antiserum equally inhibited intact cells and cell extracts. This is consistent with a great deal of evidence which indicates that phosphatase is a surface enzyme (6, 40), but does not support the idea of a surface hexokinase. Blakley & Boyer (42) found that the Michaelis constant (K_m) for glucose fermentation by intact cells was higher than the (K_m) for glucose phosphorylation by hexokinase, and that 6-fluoro-6-deoxyglucose was a more efficient inhibitor of sugar fermentation than of yeast hexokinase. Sols (43) then showed that the order of preference for glucose and fructose fermentation by intact sauterne yeast is the inverse of the order of preference for phosphorylation by cell-free extracts. He therefore suggested that an independent, stereospecific transport step preceded phosphorylation, and he interpreted Rothstein's data as evidence for a catalytic sugar carrier. Sols (44) has recently provided similar evidence that β-galactosides and α-glucosides are transported across the yeast cell membrane by a carrier mechanism before preceding their intracellular hydrolysis: (*a*) the order of preference for β-galactosides (lactose and orthonitrophenyl-β-D-galactoside) and α-glucosides (maltose and turanose) for fermentation by intact cells is the inverse of the order of preference for their hydrolysis by β-galactosidase and α-glucosidase in cell-free extracts, respectively; (*b*) galactono-γ-lactose, an efficient competitive inhibitor of β-galactosidase in cell-free preparations, does not affect the fermentation of β-galactosides by intact cells; (*c*) the pH optimum for fermentation (pH 3.5) does not correspond to the pH optimum of the hydrolytic enzymes (pH 7.0 to 7.8); (*d*) no free glucose is produced in the external medium during the fermentation of these disaccharides.

In contrast, fermentation of β-fructosides (sucrose and others) and of α-galactosides (melibiose and others) which are hydrolyzed by exoenzymes in the yeast cell wall [Friis & Ottolenghi (45, 46); Sutton *et al.* (47)] show: (*i*) the same order of substrate preference for fermentation by intact cells and hydrolysis by extracted β-fructosidase and α-galactosidase; (*ii*) the same pH optima; and (*iii*) their fermentation is associated with the release of

free hexose in the external medium during fermentation (trapped by hexokinase+ATP).

The sugar carriers, which Sols has preferred to call "transportases," are apparently constitutive transport mechanisms and, since the sugars used in his studies were all metabolizable, it is impossible to tell whether the facilitated transport is the result of facilitated diffusion or active transport. Robertson & Halvorson (48), however, had already reported that yeast α-glucoside "permease" (which was induced by maltose at the same time as the synthesis of α-glucosidase), transported the non-hydrolyzable substrate, methyl-α-D-glucoside (α-MG), and that the ability to ferment maltose decreased more rapidly than did the α-glucosidase activity in maltose-grown cells shaken aerobically in the presence of glucose. They found that the rate of deadaptation to maltose fermentation paralleled the decay of the "permease" system rather than loss of α-galactosidase, producing a transient crypticity.

Analysis of the α-glucoside "permease" by Robertson & Halvorson using α-MG showed that the transport depended upon metabolic energy; it was DNP-sensitive and resulted in accumulation against a threefold concentrated difference; it showed Michaelis-Menton kinetics (K_m for α-MG $=0.12$ M), was stereospecific, and showed competitive inhibition among certain α-glucosides. This transport would therefore be classified as an active transport. The observations of these authors and those of Sols offer convincing proof of the independence of transport and hydrolytic steps in the metabolism of α-glucosides and β-galactosides in bakers' yeast.

Avigad (49) has recently studied the genetic control of α-glucoside transport in *Saccharomyces* haploids. Lindegren (50, 51) has shown that the genes MA, MG, SU, and MZ control the inducible synthesis of specific hydrolytic enzymes for maltose, methyl-α-D-glucoside, sucrose, and melezitose, respectively. Glucose-grown cells of the following genotype, MA mg su mz, cannot ferment any of these glucosides; however, they can be induced to ferment maltose but not α-MG, sucrose, or melezitose. Avigad found that glucose-grown cells were impermeable to maltose and α-MG, but possessed a constitutive transport system for sucrose. Growth on maltose induced a specific maltose transport system and the enzyme, maltase. The constitutive sucrose transport system was unaffected. Study of another strain of the following genotype, MA MG su mz, confirmed that the sucrose system is constitutive, and showed that glucose-grown cells were impermeable to both maltose and α-MG, but that a specific transport system was induced when growth on the individual glucoside induced the corresponding glucosidase. Thus, while sucrose transport was found in the absence of sucrase activity, maltose and α-MG transport were induced simultaneously with the corresponding glucosidase. In this latter respect, these studies confirm the observations of Robertson & Halvorson (48) on the induced maltose fermentation of a genetically undefined yeast.

Monosaccharide transport.—Evidence for a carrier-mediated transport of

monosaccharides in bakers' yeast has come largely from studies with non-metabolizable sugars. Burger *et al.* (9), Cirillo (7, 25, 26), and Kotyk (27) have shown that a variety of non-metabolizable sugars are transported across the yeast cell membrane, contrary to earlier reports. Cirillo found that the following non-metabolizable sugars were transported by a stereospecific carrier system: L-sorbose, L-galactose, D-xylose, D-arabinose, L-fucose, L-arabinose, D-xylose, D-ribose (in decreasing order of efficiency).

In this system, transport was independent of metabolic energy and there was no accumulation against a concentration difference. The transport showed Michaelis-Menten kinetics (the K_m for sorbose is *ca*, 0.2 M) and was stereospecific. The transported monosaccharides showed competitive inhibition and sugar transport was selectively inhibited by uranyl ion.

The monosaccharide transport system is therefore a facilitated diffusion. Since the transported sugars are not metabolizable by bakers' yeast (i.e., only galactose can be metabolized after induction) and some, like sorbose, are not hexokinase substrates [Sols (52)], transport appears to be independent of hexokinase activity. This has been demonstrated more directly by showing that sorbose uptake continues without an external energy donor, under a nitrogen atmosphere and with $10^{-3} M$ iodoacetate. Under these conditions, phosphate metabolism measured in the presence of glucose was completely blocked, i.e., glucose metabolism was blocked and esterification of either intracellular or extracellular inorganic phosphate was totally in-inhibited [Cirillo (26)] and the amount of sorbose transported, on a molar basis, was 10 times the total organic and inorganic phosphate of the cell. Furthermore, free glucose, absent in uninhibited cells, is present in the cell at a level equivalent to that of a non-metabolizable sugar at the same extracellular concentration.

The transport system studied with non-metabolized sugars seems to be the common system for both metabolizable and non-metabolizable sugars since glucose and the non-metabolizable sugars show competition for transport; uranyl ion inhibits glucose utilization and sorbose transport to the same degree; and counterflow occurs between glucose and non-metabolizable sugars. Counterflow is the exchange reaction between an external sugar and an internal sugar, causing the internal sugar to move out of the cell against a concentration difference. Counterflow is demonstrated experimentally by equilibrating cells with one sugar and then adding a second sugar to the external medium. If the two sugars use a common carrier, and if the sugar-carrier complex can be formed independently on the two sides of the membrane, the internal sugar may be moved out of the cell against a concentration difference [see Rosenberg & Wilbrandt (53) for a full treatment of counterflow]. Counterflow of sugars has been described in erythrocytes and in heart muscle [Park *et al.* (54, 55)].

The mechanism of uranyl ion inhibition of sugar transport was postulated by Rothstein to be an inhibition of sugar phosphorylation upon which sugar transport was presumed to depend. It is now clear that uranyl ion

inhibits the operation of the carrier system. Rothstein had already suggested that uranyl ion combines with polyphosphate groups in the cell membrane. The recent results reported by Few *et al.* (56) which identify phosphatidic acids as the polyphosphate groups for which uranyl ion has very great affinity, is very suggestive since recent studies of the Hokin's (57, 58) have implicated phosphatidic acids as carriers in several active transport systems. Whether microbial phosphatidic acids indeed serve as carrier components in facilitated diffusion is worth further study.

While all of the recent work done on sugar transport in yeast agrees as to the role of stereospecific membrane components, the work done by Robertson & Halvorson (48) and by Avigad (49) on disaccharide transport differs from that reported on monosaccharides with respect to energy dependence. Disaccharide transport has been reported to be dependent upon metabolic energy while monosaccharide transport is not. Since energy dependence for disaccharide transport was determined by means of DNP sensitivity, the possibility of non-specific toxicity at concentrations higher than necessary required to uncouple phosphorylations might account for this discrepancy. Since sucrose transport did not result in accumulation against a concentration difference, and α-MG was accumulated against a relatively small concentration difference (*ca.* threefold), the conclusion that these transports arise from an active process should be made with caution.

E. Coli Permeases

In contrast to sugar transport in yeast, sugar transport in *Escherichia coli* is an active transport, capable of accumulating sugar against great concentration differences. Evidence supporting active transport of a number of sugars and sugar derivatives was reviewed by Cohen & Monod (59); they developed the general theme of a stereospecific membrane-transport process in microorganisms. They noted that sugar transport in *E. coli* required metabolic energy and was astonishingly efficient, accumulating the unchanged sugars against 100- to 10,000-fold concentration differences. The process was stereospecific with structural analogues competing for entry. A competitive sugar caused the displacement of a previously accumulated sugar at a rate, and to an extent, consistent with its relative affinity for the transporting system.

From work on inducible and selected mutant strains, and the use of non-metabolizable thiogalactosides, they showed further that the transport system was independent of the presence of the hydrolytic enzymes necessary to initiate their metabolism (i.e., β-galactosidase) and its induction shows all of the characteristics of induced protein synthesis.

Since β-galactoside transport depends upon a specific protein, and the transport protein exhibits the enzyme-like properties inferred from saturation kinetics, stereospecificity, competitive inhibition, and sensitivity to enzyme inhibitors, Rickenberg *et al.* (60) coined the term "permease" to describe this system. An independent description of the active transport of

β-galactosides in *E. coli* was also made by Pardee (61), and after these reports, many papers on "permeases" have appeared. In 1957, Cohen & Monod (59) presented a model to explain the active transport of sugars and amino acids. The original model envisaged a stereospecific, catalyzed entry step resulting in accumulation against a concentration difference, and a non-specific exit reaction through which the accumulated sugar "leaks" by simple diffusion. The intracellular concentration would be determined by the ratio of the rate of entry and rate of exit.

Before considering some of the recent aspects of this work, reference should be made to the use of β-thiogalactosides as non-metabolizable substates of β-galactoside transport. Rotman (62) has shown that β-thiogalactosides are transported by a transport system independent of that used by galactosides; the latter are transported by a constitutive permease while the former are transported by an inducible one. One should be aware that the results obtained with β-thiogalactosides, while paralleling the results obtained with β-galactosides, may not be identical.

The recent observations of Kepes (63) and Horecker *et al.* (64, 65, 66) have contributed notably to the original model of sugar permeases. They showed that the exit reaction is not a non-specific leak, but an inducible facilitated diffusion system. Any new model must, therefore, incorporate stereospecific mechanisms for both the entry and the exit reactions.The requirement for a specific exit process has also been indicated by the observations of Wiesmeyer & Cohn (67) on a maltose-concentrating system; their mutant strain of *E. coli* could accumulate maltose up to 25 per cent of the dry weight of the cells, but the accumulated sugar was not lost to a sugar-free medium. The extremely high internal concentrations that are reached in this system are undoubtedly caused by a lack of exit reaction.

Cohen & Monod (59) considered that the *E. coli* permeases could be visualized either as an energy-dependent entry reaction or as a two-step process consisting of an energy-independent entry reaction plus an energy-dependent accumulation reaction. Horecker and his co-workers (66) have recently presented evidence supporting the latter suggestion: studying galactose accumulation by a galactokinase-less strain of *E. coli*, they showed that the entry reaction is not energy-dependent and continues at an undiminished rate when the accumulation process is inhibited by DNP. The previously described inhibition of galactose accumulation by this strain (64, 65) has been shown to be ascribable to an increased exit rate rather than an inhibition of the entry reaction.

From these recent developments, the following overall process emerges: (*a*) an energy-independent entry by facilitated diffusion; (*b*) an intracellular, energy-dependent accumulation process; and (*c*) an energy-independent exit by facilitated diffusion.

The nature of the energy-dependent reaction and its localization in the cell can only be speculated upon. The situation is similar to that encountered by Gale in amino acid transport and accumulation in staphylococci, with

the exception that in that case the entry and exit reactions were described as arising from simple diffusion rather than facilitated transport. Faced with this picture, Gale (85) was prompted to write:

A botanist presented with a situation similar to that discussed here might suggest that free diffusion . . . occurs into the internal medium of the cell; when [an energy source] is available, active transport into and accumulation within a vacuole takes place. If this is true, then our problem is merely removed from the door step to the serving hatch, but for the present there would seem little point in postulating a hole in a bacterium to explain a hole in a postulate.

Heeding this admonition, one could visualize the energy-dependent accumulation reaction to be the result of an energy-dependent complex formation between the transported sugar and some specific receptor sites within the cell. This mechanism has indeed been invoked by Bolton *et al.* (68, 69) to explain amino acid accumulation in *E. coli*. However, the large amounts of sugar accumulated and the demonstration by Sistrom that the accumulated β-galactosides are osmotically active make this explanation unsatisfactory. The alternative to this "stoichiometric site" hypothesis is the possibility of intracellular "osmotic regions" with different permeability "characteristics" or "accessibilities" as suggested by Cohen & Monod and Bolton *et al.* These osmotic regions would, in effect, be the "holes" mentioned by Gale. However, numerous data can be cited to support the belief that intracellular compartmentalization exists in bacteria, yeasts, and molds. Such compartmentalization may be involved in the separation of osmo-sensitive expandable and the osmo-resistant amino acid pools of yeast and bacteria [Cowie & McClure (70); Britten & McClure (71); Roberts *et al.* (72); Halvorson & Cowie (73)].

There are several instances in which destruction of the outer cell membrane does not destroy permeability barriers in the interior of the cell. Foulkes (74), for example, showed that whole yeast was impermeable to citrate—presumably the reason intact yeast could not oxidize this compound. Frozen and thawed cells still could not oxidize citrate although the yeast cell was now quite permeable to citrate. Only when freezing and thawing was followed by chloroform extraction was citrate oxidized. Rothstein *et al.* (75) described a similar situation for yeast fermentation of fructose diphosphate (FDP). Whole cells fermented only glucose; FDP presumably could not be fermented because of the impermeability of the cell membrane. However, acetone-extracted dried yeast did not ferment this diphosphate ester although glucose was actively metabolized. On the other hand, soluble extracts are well known to be able to ferment FDP. Finally, Eggleston & Krebs (76) reported that an *E. coli* mutant unable to ferment free ribose, did ferment ribose when the latter was attached to purines; presumably it entered the cell via a purine transport system. Frozen and thawed cells could ferment ribose only in ribosidic or ribotidic form even though freezing and thawing was shown to destroy the permeability barrier to amino acids. Only sonicated preparations could ferment free ribose.

These examples illustrate the possible role of intracellular osmotic barriers in accumulation of sugars and other metabolites. However, it must be pointed out that the types of intracellular compartmentalization which exists in cells of higher forms, for example, the presence of endoplasmic reticulum [Palade (77); Siekevitz (78)] have been shown only in yeasts and molds which also have well-developed vacuoles (79 to 82). There is no evidence to support the presence of internal membranes demonstrable by electron microscopy for the Eubacteriaceae [Bradfield (83); Murray (84)]. For the present, then, there is only indirect evidence for the presence of intracellular "osmotic regions."

Sequential utilization of substrates.—A number of instances of sequential utilization of certain sugars which the organism can use immediately and without adaptation when they are present alone, seem to be the result of competition for a common transport system. Kleinzeller *et al.* (86) showed that ribose-grown *Rhodotorula* fermented glucose in preference to ribose when these sugars were present together, even though it used ribose without a lag when alone. Cirillo & Grayson (87) compared the pattern of sugar utilization by *Tricholoma nudum* of three sugars. When present singly, mannose, glucose, and xylose were used immediately. However, when all three sugars were present together, the order of utilization was mannose, glucose, xylose. Essentially identical results were obtained with resting cell suspensions. Since free sugars never accumulated in the cells during utilization, it was suspected that transport is the rate-limiting step and that competition occurred at the entry step because the order of utilization of mannose, glucose, and xylose correlated with their individual affinity (i.e., the inverse of the Michaelis constant determined for each sugar alone) for the transport process. The preference for glucose over fructose by this basidiomycete was also demonstrated by Reusser *et al.* (88) who recovered almost pure fructose in high yield from the action of *Tricholoma* mycelium on sucrose solutions. A similar glucose-fructose competition has long been known for bakers' yeast, and Friis & Ottolenghi (45, 46) were able to prevent the utilization of glucose produced by the extracellular hydrolysis of sucrose and melibiose by adding large amounts of fructose to the mixture. Such competitive effects between two substrates, when one of the substrates is used only after enzymatic induction (such as galactose in bakers' yeast and *Tricholoma*), may account, at least in part, for the diauxie phenomenon. In this case, the constitutive substrates prevent uptake of the inducer, and induction cannot occur until the constitutive substrate is consumed [Burger *et al.* (9)].

The Mechanism of Facilitated Transport

Active transport will first be considered as two separate processes: (*a*) a facilitated diffusion, (*b*) an energy-dependent accumulation process. The justification for this suggestion has been given above and discussed by many authors [Crane (12); Le Fevre (19); Widdas (20); Bowyer (23); Rosenberg & Wilbrandt (53, 89)].

Facilitated diffusion.—The comprehensive review by Bowyer (23) discusses the models for facilitated diffusion of non-electrolytes. None of the recent work done with microorganisms suggests new models, nor does the microbiological work done so far permit choice among the models. The trio of (*a*) saturation kinetics, (*b*) stereospecificity, and (*c*) competitive inhibition of facilitated transport, however, can only be explained if a carrier or an enzyme, or both, are involved in the transport process [Rosenberg & Wilbrandt (89)].

The schemes presented in Figure 1 show, in a general way, some of the most commonly discussed models [Rosenberg & Wilbrandt (89)]. Schemes 1 and 2 represent the membrane-carrier hypothesis in which the substrate, (S), combines with carrier, (C), at the outer surface of the cell to form a carrier-substrate complex, (CS). In scheme 1, the CS complex is formed from either side of the membrane and the carrier is a fixed point in the membrane. In scheme 2 the carrier and CS complex are mobile components which, in any number of ways [Danielli (24)], reach the inner surface of the cell where the substrate is released. The rate of transport at saturation is limited either by the equilibrium constant of the CS complex or, if the carrier is mobile, by the rates of diffusion of the free carrier or the CS complex. In scheme 3, a membrane enzyme converts a membrane-insoluble substance, S, into a membrane-soluble product, T. The product then diffuses through the membrane, and by a reversal of the enzymatic process, the original membrane-insoluble substance is released. Scheme 4 represents a modification of the carrier model in which substrate carrier complex formation does not form spontaneously but depends on an enzymatic reaction.

In all of these schemes the saturation kinetics are a function of either the equilibrium constants of the various complexes (CS, ES, CES) which are formed during transport or the rate of diffusion of any mobile components through the membrane.

Since all of the models described in Figure 1 can result in saturation kinetics when the concentrations of substrate are sufficiently high to exceed the "Michaelis" constants of the appropriate complexes, the presence of saturation kinetics alone cannot decide the appropriate model. In fact, the absence of saturation kinetics also does not exclude any of these models since non-saturating kinetics are observed if the concentration of substrate does not exceed the appropriate equilibrium constants. [A thorough analysis of the kinetics of these and other models is presented by Bowyer (23).]

The choice among these models must be made on the basis of other criteria. The participation of a protein in sugar transport has been demonstrated most convincingly in the inducible "permeases" and the exit reactions of sugar transport in *E. coli* [Cohen & Monod (59); Kepes (63); Horecker *et al.* (64, 65, 66)]. However, it is not at all clear what role this protein plays. The protein may itself be the carrier of scheme 1 or 2; in this case it also confers the specificity on the transport process. In schemes 3 and 4, the protein acts as a classical enzyme. Therefore, the evidence of the

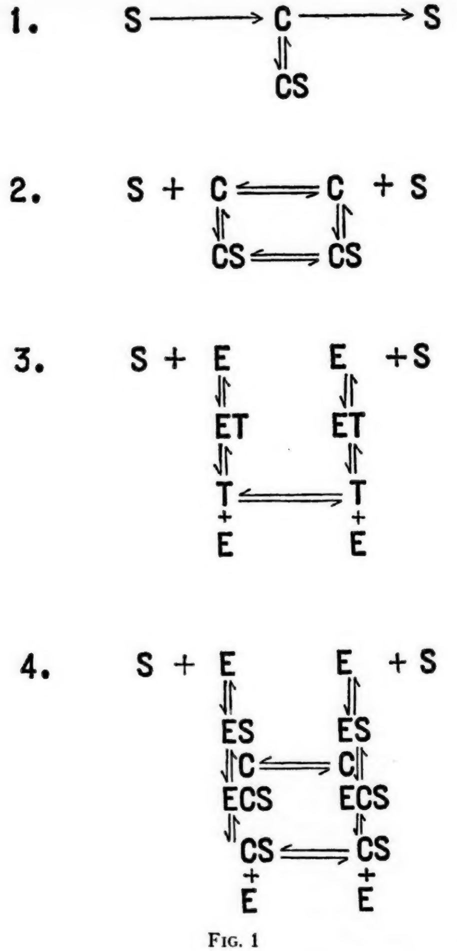

FIG. 1

participation of a protein in a given transport does not necessarily aid in choosing among the suggested models. However, a choice is possible between the fixed carrier and mobile carrier hypothesis.

The mobile carrier or "ferry boat" hypothesis predicts that, under appropriate conditions, the movement of one sugar can bring about uphill transport of a second sugar in a cell normally unable to move sugars against a concentration difference. This phenomenon, called "counterflow," has

been demonstrated to occur in sugar transport in yeast [Burger *et al.* (9); Cirillo (25, 26)]. It suggests that a carrier mechanism must be involved in the transport process. This would require a choice between schemes 2 and 4. The resemblance of many features of sugar transport to enzymatic reactions as well as susceptibility to enzyme inhibitors and their inducibility, would suggest scheme 4, the enzymatic carrier model, as a satisfactory minimal model to explain all of the present data.

The possibility that active transport is a two-step process in which an energy-independent entry process is followed by an energy-dependent process within the cell, has been developed above. While such a two-step process has the appeal of providing a unitarian concept for the entry of both passive and active processes, the possibility of energy-dependent transport must also be considered. Furthermore, even if the entry process in both types of transport is energy-independent facilitated diffusion, the mechanism of energy-coupled transport across intracellular membranes must still be considered. Of the various models suggested to explain coupled transport, the metabolic link hypothesis [Mitchell (90, 91)] in any of its variations provides the best model. According to this mechanism scheme, transport is carried out by group-transfer metabolic enzymes in the cell membrane by means of which transport is linked to the first step in the metabolic utilization of the substrate. Hexokinase, for example, which carries out the group-transfer reaction, ATP+glucose=glucose−6-ph+ADP, could bring about glucose transfer into the cell if: (*a*) "the active centre of the enzyme system (e.g., hexokinase) was anistotropic so that the group donor (e.g., ATP) must react only from one side while the group acceptor (e.g., glucose) must react from the opposite side; and (*b*) the enzyme molecules are appropriately oriented in the osmotic barrier" [Mitchell (90), p. 428)]. This hypothesis is generalized in Figure 2A.

The evidence for the independence of transport mechanisms and metabolic capacity reviewed above seems to argue against the metabolic-link hypothesis in spite of its apparent biological economy of having a single step for transport and initiating metabolism. Mitchell, however, has argued that a demonstration of the separate occurrence of transport and enzymatic capacity in certain mutants may be only apparent and does not necessarily exclude the metabolic link hypothesis as a general mechanism.

He suggests that the synthesis of the enzyme and its location in the cell membrane (which is the only place in which it can act in transport mechanisms) are under separate genetic control. Therefore, its synthesis and membrane location would be expected to be independently mutable and inducible, and the separability of transport and metabolism would be the result of separate effects on enzyme synthesis and membrane location. While this suggestion may explain the occurrence of cryptics, it does not readily explain the ability of cells to transport sugars which are not substrates of the proposed enzyme, e.g., transport of the non-metabolizable sugars by bakers' yeast or the ability of galactokinase-less *E. coli* to transport galactose. Here,

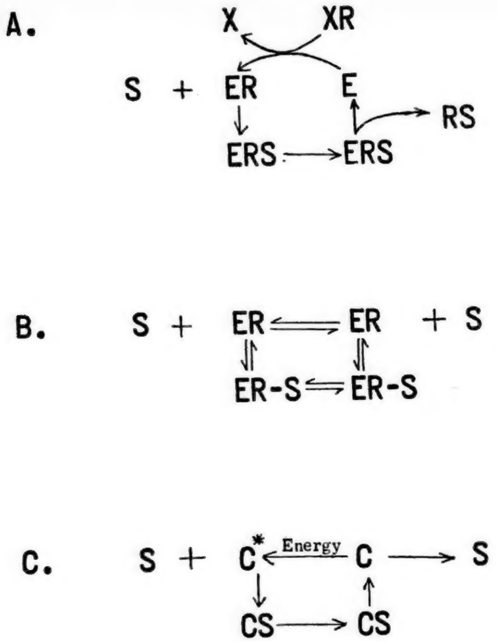

Fig. 2

the substrates obviously are being transported despite the absence of the group-transfer reaction upon which this transport is supposed to depend.

Burger *et al.* (9) have attempted to answer this anomaly. They suggest that while the group-transferring enzyme is responsible for the transport, the group-transfer reaction itself is not necessary. They suggested, for example, that hexokinase, probably in its phosphorylated form, may serve as the carrier molecule, but without carrying out its phosphorylative role. This suggestion was made to explain the fact that, in sugar-transport in yeast, the relative affinity of the sugars in the transport process is similar to the relative affinity of the sugars for hexokinase (See Fig. 2B).

This suggestion has a serious shortcoming. Although the relative affinity for the transport process of the metabolizable sugars as a group, and of the non-metabolizable sugars as a group, corresponds with their affinity for hexokinase, the affinities of the individual sugars do not [Sols (52)]. Sorbose, for example, is not a substrate of yeast hexokinase and shows no affinity for the enzyme at any concentration, yet it is transported quite readily by bakers' yeast.

By present knowledge, then, the metabolic link hypothesis, in spite of

its inherent attractiveness, seems wanting in ability to explain a large number of transport data. The best working hypothesis for sugar transport across the cell membrane is that it is an energy-independent facilitated diffusion since entry per se seems to be energy-independent.

The accumulation step, however, may involve energy-dependent carrier transport across intracellular membranes. Such a transport may be mediated either by an energy-linked transport of the generalized form shown in Figure 2C in which the carrier, C, requires an energy-dependent activation to C* in which form it transports its substrate across the membrane. This last model is the most general form of energy-dependent membrane processes whether it occurs at intracellular membranes or at the cell surface. The suggestion of an energy-independent facilitated diffusion at the cell surface followed by an energy-dependent transport across intracellular membranes can easily explain the overall characteristics of sugar transport in yeast and *E. coli.*

Implicit in the carrier and the metabolic link hypotheses discussed so far is the concept that the carrier or the metabolic linking enzymes are present in an otherwise impermeabile or poorly permeable cell membrane. However, active transport processes which result in accumulation against a concentration difference, would produce indistinguishable results if the energy-dependent accumulation process were the result of simple diffusion through a permeable membrane followed by a metabolically dependent complex formation between the substrate and specific receptor sites in the cells.

All of the major features of active transport described above, namely, metabolic dependence, accumulation against a gradient non-linear kinetics, stereospecificity, competition, and sensitivity to certain inhibitors can be explained either on the basis of the bulk properties of protoplasm or as the result of membrane phenomena. Several hypotheses have been developed to explain the accumulation phenomena in animal cells [Baird *et al.* (92); Szent-Gyorgyi (93, 94); Kurella (95)], plant tissue [Briggs & Robertson (96)], and in bacteria on a basis other than membranes. The concept of permeability being the result of "sorption" forces (i.e., solution, adsorption, and binding by protoplasm as a bulk phase) has been developed by several authors [see Ungar (97); Troshin (98)]. However, convincing evidence has been presented by many authors to preserve the classical concept of a selectively permeable membrane at the cell surface responsible for most of the permeability phenomena of the cell. Reviews by Mitchell & Moyle (32, 33), McQuillen (99), and Weibull (100, 101) have presented data which support the view that almost all of the low-molecular constituents of the cell are free and osmotically active. The recent literature continues to support this classical picture (102 to 105). Chemical analyses of isolated protoplast membranes have also supported the picture of the membrane presented by Danielli for mammalian cells [see Gilby and his colleagues, Few and McQuillen (106, 107)].

A possible mechanism to explain accumulation that has not previously

been discussed is the recent finding that the active transport of sugars across intestinal mucosa may be coupled to an energy-dependent, sodium-extrusion process (i.e., a sodium pump) [Crane (12)]. A strikingly similar observation has been made by MacLeod & Hori (108) and by Payne (109) who have shown a requirement for both sodium and potassium in the utilization of tricarboxylic acids and glucuronate in marine bacteria. The sodium dependence for the utilization of these substrates seems to be associated with a transport process since cell-free enzyme preparations showed no such sodium requirement for the metabolism of these substrates. As a result, MacLeod & Hori have suggested a possible dependence on a sodium pump. The possibility of substrate transport linked to a "sodium" or any other ion pump analogous to that in intestinal sugar transport, offers a basis for the accumulation process which should be further explored.

The nature of the carrier.—Aside from identification of the sugar carrier as the enzyme which carries out the first step in metabolism suggested by the metabolic link hypothesis, the nature of the carrier molecule embodied in other hypotheses is uncertain. The stereospecificity requires a kind of structure usually associated with proteins and nucleic acids. However, the participation of lipid membrane constituents should not be neglected. While the lipid components of the cell membrane have usually been considered to be important from a structural point of view, evidence of phosphatidic acid as a dynamic participant in transport has been presented by Hokin & Hokin (57, 58) in a variety of secretory activities. A possible role of phosphatidic acids in sugar transport is suggested by the uranyl ion inhibition of sugar transport in yeast and molds [Cochrane & Tull (8); Cirillo (25, 26); Rothstein (40)] and amino acid transport in staphylococci [Gale (85)]. Few *et al.* (56) have found that phosphatidic acids are the constituents of bacterial cell membranes for which uranyl ion has the greatest affinity. The specific inhibitory activity of uranyl ion on sugar and amino acid transport may suggest an important role for phosphatidic acids as a component of the membrane carriers.

Recently, Wiesmeyer & Cohn (67) have shown that in *E. coli* ability to absorb *lambda* phage and the presence of maltose permease seem linked: cells which lack the maltose permease lack the phage receptor site. They suggest that the virus reception site and the "permease" are "closely, physically associated. If this were true, a rather direct approach to the isolation and chemical characterization of 'permease' might be available."

Amino acid transport.—Only a few aspects of amino acid transport will be referred to which bear on the question of the dependence of amino acid transport on activating enzymes in the cell membrane, and on the nature of the amino acid carriers. The ability of yeast and bacteria to concentrate amino acids against large concentration differences by an active process is well documented [Roberts *et al.*, (72); Gale, (85)]. Cohen & Monod have reviewed the evidence favoring the interpretation that amino acid transport involves stereospecific membrane carriers analogous to those discussed for sugars. The evidence against the application of the metabolic link hypoth-

esis to amino acid transport is of two types. First, the general observation from many studies that the substrate specificity of the transport process is not the same as that of the activating enzymes, e.g., Cohen & Rickenberg (110) showed that a number of structurally unrelated amino acids are transported by the same system in *E. coli*. Lark (111) showed that although the methionine transport system of *Alcaligenes faecalis* is specific for this amino acid, it transports both the D and L isomer. Finally, Halvorson & Cowie (73) have also shown that the amino acid transport systems in yeast can transport structurally unrelated amino acids although the specificity is narrower than that of *E. coli*. The second type of evidence against the participation of activating enzymes in amino acid transport is the fact that there is no increase in phosphate uptake when large amounts of amino acids are accumulated both in yeast and bacteria (72, 73). In fact, the concentration of amino acid which is accumulated greatly exceeds the total pool phosphorous.

The nature of the amino acid pools in Gram-negative bacteria and yeast also suggest that the transport is independent of amino acid activation. Two functionally and physically separate pools have been demonstrated in these organisms: (*a*) an osmo-sensititive, expandable pool which is in equilibrium with the external amino acids, and which reflects the concentration and the ratio of the amino acids in the external medium, and (*b*) the osmo-resistant, internal pool in which the amino acid concentration is constant, and in which the amino acid ratio is identical with that of cellular protein. Amino acids from the external medium are transported into the expandable pool from which they are subsequently transferred to the internal pool and from there into protein. Only when the external amino acid concentration is very high can amino acids be incorporated into protein without passing through the internal pool [Halvorson & Cowie (73)]. The presence of these separate pools reflects the independence of transport and amino acid activation preparatory to protein incorporation. The presence of independent mechanisms, rather than being an "extravagant" duplication [see Mitchell (90)], permits the organism to accumulate individual amino acids at concentrations and in ratios determined by their availability in the external medium, independent of the more restrictive capacity of protein synthesis.

Unlike the sugar carriers, amino acid transport is mediated exclusively by constitutive systems; however the recent report by Vogel (112) has shown that the phenomenon of "repression," whereby the final product of an enzymatic sequence may inhibit the synthesis of early members of the chain [Vogel (112)], applies also to the permeating mechanism by which external substrates reach the intracellular enzymes. Arginine has been shown not only to repress the synthesis of *N*-acetylornithase, but also the *N*-acetylornithine "permease" by means of which its substrate enters the cell. This illustrates the elegant control exercised by the cell not only of its intracellular enzymes, but also of the permeation mechanisms which feed into them. By means of inducible and repressible permeases, then, the cell can change its spots and permit survival in different environments.

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STRUCTURE OF VIRUSES[1,2]

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INTRODUCTION

Some understanding of the molecular structure of the small and medium sized viruses and of the significance of their various components in the infectious process—from attachment and penetration to self-replication—has been gained only within the last decade. Contemporary investigations have emphasized the different and separable roles of the protein and nucleic acid moieties which are the essence of the virus particle. Information relating to the structure of the viral protein coat at the level of macromolecular organization is substantial; and, since our understanding of the mechanisms by which viral components are synthesized and then welded together into the mature infectious particle depends in part on a knowledge of the actual organization and orientation of the viral materials, it would seem profitable to summarize here recent observations on viral fine structure. The importance of information regarding the structure and activity of viral nucleic acids nees no emphasis, and a discussion of the present position of those investigating this aspect of virus structure needs no justification.

PHYSICAL PROPERTIES

We have not undertaken to summarize here the extensive literature regarding such physical properties of viruses as length, diameter, sedimentation velocity, diffusion constant, etc., but have elected instead to concentrate on the rapidly expanding body of evidence concerning the fine structure(both surface and internal) of virus particles. Although plant viruses have been most widely studied from this point of view, there is now considerable information regarding mammalian, insect, and bacterial viruses as well. Much of the early data regarding fine structure came from x-ray diffraction studies, though it should be noted that evidence confirming the existence of subunits was often provided by chemical analysis. In the last few years, the development of newer, more refined techniques has made it possible for the electron microscopists to make major contributions to our knowledge of this subject.

[1] The survey of the literature pertaining to this review was concluded in January, 1961.

[2] The following abbreviations will be used: BSV (bushy stunt virus); DNA, DNase (deoxyribonucleic acid, deoxyribonuclease); ECHO (enteric cytopathogenic human orphan); EEE (Eastern equine encephalitis); NDV (Newcastle disease virus); PBS (phosphate-buffered saline); RNA, RNase (ribonucleic acid, ribonuclease); SBMV (Southern bean mosaic virus); TMV (tobacco mosaic virus); TRV (tobacco rattle virus); TYMV (turnip yellow mosaic virus); WEE (Western equine encephalitis).

It seems appropriate at the outset to recall that in 1956, Crick & Watson (1) postulated that all small viruses—plant or animal, spherical or rod-shaped—are composed of a large number of identical protein subunits packed together in a regular manner around the viral nucleic acid. They suggested further that all small, spherical viruses would have cubic symmetry (of the tetrahedral, octahedral, or icosahedral type); that the number of subunits present would be a multiple of 12; and that this hypothesis would be valid for viruses containing deoxyribonucleic acid (DNA) as well as for those containing ribonucleic acid (RNA). The fact that, of the polyhedrons considered, the icosahedron has the largest surface area to volume ratio, and therefore approximates most closely to a sphere, prompted Crick & Watson to predict that the small spherical viruses would have the symmetry (532 cubic symmetry), though not necessarily the shape, of this polyhedron. It should be remembered that at the time this theory was advanced, there was conclusive supporting evidence in the case of two viruses only, the rod-shaped tobacco mosaic (TMV) and the spherical tomato bushy stunt (BSV) viruses. The remarkable extent to which their predictions have been supported by subsequent experimentation will be clear from what follows.

Plant Viruses

Tobacco mosaic virus (TMV).—No virus has been as extensively studied as the rod-shaped TMV, and no virus illustrates more clearly the subunit principle in virus structure. Its over-all dimensions have been reliably established by electron microscopy to be 300 $m\mu$ (length)$\times$15 $m\mu$ (mean diameter) [Williams & Steere (2); Hall (3)], and its molecular weight has been estimated by Boedtker & Simmons (4) to be $39 \pm 1 \times 10^6$.

The early x-ray studies done by Bernal & Fankuchen (5) showed clearly that the TMV particle had some sort of subunit structure, and the observations of Watson (6) and of Franklin (7) suggested that the arrangement of the subunits was helical. Chemical evidence that indicated the presence of identical subunits in TMV was provided at about the same time by Harris & Knight (8, 9). They showed that incubation of TMV protein with carboxypeptidase resulted in the release of only one amino acid, threonine. From the amount of threonine released they estimated that the TMV particle contained some 2500 polypeptide chains, all terminating in threonine at the carboxyl end, and having a molecular weight of about 18,000.

Subsequently, the extensive x-ray diffraction studies carried out by Franklin and her collaborators (10 to 13) led to a detailed picture of the structure of this virus. The particle is built up of 2130 identical protein molecules (of molecular weight about 18,000) arranged with RNA in a hollow spiral with a pitch of 2.3 $m\mu$. Three turns of the spiral contain 49 of the subunits. The central hole of the particle has a diameter of 4 $m\mu$ and the RNA, which is embedded in the protein, follows a helical path in the rod at a radius of 4 $m\mu$.

The existence of the hollow core, deduced from x-ray diffraction analysis,

has been shown by Huxley (14) and by Brenner & Horne (15) in electron micrographs. Recently, Nixon & Woods (16), making use of the negative staining technique of Brenner & Horne (15) have obtained electron microscope images of reaggregated TMV protein which provide visual confirmation of the proposed structure of this virus. Finally, direct experimental evidence for the existence of a native subunit having a molecular weight of 18,000 has been obtained by Ansevin & Lauffer (17) who succeeded in dissociating TMV protein into undenatured particles with a sedimentation coefficient consistent with that molecular weight.

Tobacco rattle virus (*TRV*).—This rod-shaped virus, which resembles TMV in general morphology but is serologically unrelated to it, has received relatively little attention from investigators of virus structure. Although it has not been the subject of definitive x-ray diffraction studies, recent electron microscopic investigations have yielded results which seem worth recording here.

Harrison & Nixon (18) have described the separation and partial purification of TRV from infected tobacco leaves. They found that the particles, all of which were 25 mμ wide, were 73 to 77 mμ or 179 to 192 mμ long—values which are in good agreement with those previously reported by Paul & Bode (19). Although only the longer particles are infectious, the two classes show no substantial differences in serological behavior, electrophoretic mobility ultraviolet absorption spectrum, or gross chemical composition.

From electron microscope studies of these preparations, Nixon & Harrison (20) have shown that the particles are tubular, with a central hole 4 to 6 mμ in diameter and an outside diameter of 17 to 25 mμ, depending on the treatment. Particles stained with uranyl acetate, phosphomolybdic and phosphotungstic acids exhibited transverse bands 2.5 mμ apart. They suggest that these bands may represent the protein helix of the particle, a suggestion which seems quite justified in the light of the investigations of Nixon & Woods (16) discussed previously. Although no direct evidence regarding the position of the nucleic acid in the particle was obtained, the electron microscope images suggest that it may occupy a position 5 mμ from the center. The helical arrangement implies a subunit structure, and although end-group analyses, which could confirm this picture, have not been done, there seems no reason to doubt seriously the suggestion made by Nixon & Harrison that the fine structures of TRV and TMV are essentially the same.

Bushy stunt virus (*BSV*).—The x-ray diffraction studies performed by Caspar (21) on crystals of this agent provided the first confirmation of the prediction made by Crick & Watson (1) regarding the structure of small spherical viruses. The data showed that the virus particle had the point group symmetry 532, from which Caspar suggested that it was constructed of 60 (or a multiple of 60) structurally identical asymmetric subunits. Taking the molecular weight of BSV as 9×10^6 [Williams & Backus (22)], its RNA content as 17 per cent [deFremery & Knight (23)], and assuming 60 subunits, the maximum molecular weight of each unit would be 125,000.

Recently, Niu, Shore & Knight (24) have analysed BSV for amino- and carboxy-terminal amino acid residues. No free amino-terminal groups could be detected, an observation which has been made with TMV by a number of investigators [Harris & Knight (9); Schramm *et al.* (25); Fraenkel-Conrat & Singer (26)]. However, the C-terminal residue was found by hydrazinolysis [Akabori *et al.* (27); Niu & Fraenkel-Conrat (28)] to be leucine, and, from the number of leucine residues liberated per unit weight of virus, the subunit weight was estimated to be 57,000 (or 48,000 when the contribution of the nucleic acid was deducted).

Hersh & Schachman (29) have disrupted bushy stunt virus with sodium dodecylsulfate and have separated and purified the slowly sedimenting material generated in this way. The molecular weight of this protein, which the authors considered to be the molecular subunits of the virus, was found to be 60,000. This value is roughly half that advanced by Caspar as the maximum molecular weight of the subunit, and is in fair agreement with the value proposed by Niu *et al.* Hersh & Schachman suggest that bushy stunt virus is composed of 120 of these units together with the RNA.

Turnip yellow mosaic virus (*TYMV*).—This small spherical virus (diameter: 28 to 30 mμ) has been extensively studied by a variety of techniques. It was first suggested by Markham (30) that the protein part of the particle was in the form of a spherical shell enclosing a core of RNA, a picture later confirmed by Schmidt and co-workers (31). Electron micrographs made by Cosslett (32), Steere (33), and, more recently, by Brenner & Horne (15), suggested that the virus is constructed of regular subunits. Klug and his associates (34, 35), on the basis of extensive x-ray diffraction studies, concluded that the subunit had a diameter of approximately 6 mμ, and that their x-ray diffraction patterns could be most readily explained if the protein shell were constructed of 60 such units, arranged 5 per face of a dodecahedron.

The recent electron microscope studies of Huxley & Zubay (36) and of Nixon & Gibbs (37) have shown that the model deduced from x-ray diffraction studies does not even approximate the facts. The protein shell of the TYMV particle is actually composed of 32 structural subunits. Huxley & Zubay postulate that these units are arranged at or near the vertices of either a pentakis dodecahedron or a rhombic triacontahedron. These are both polyhedra having 32 vertices and 60 identical triangular faces and 30 identical rhombic faces, respectively. These investigators point out that such an arrangement would explain the 532 symmetry suggested by the x-ray diffraction patterns of Klug *et al.* They note, in addition, that if the subunits were roughtly spherical or cylindrical, there would be 60 holes in their model, and the spacing between them would be 5.5 to 6.0 mμ. This value corresponds very well to the subunit size suggested by Klug and his associates.

There are, to our knowledge, no published results of end-group analyses in the protein of TYMV. However, Brenner (38) has reported that Hindley & Harris have demonstrated the presence of a protein subunit in the virus

of molecular weight about 20,000, and Markham (39) has indicated that the complete amino acid analysis carried out on TYMV by Roberts & Ramasarma (40) suggests a subunit size in the vicinity of 20,000. The virus has a molecular weight of about 5×10^6 and contains 37 per cent RNA. The protein part would then have a "molecular weight" of 3.2×10^6 which would correspond to 160 chemical subunits per virus particle, or an average of 5 chemical units per structural subunit. Huxley & Zubay (36) report that the subunits seen in their electron microscope images often appear ring-shaped, and point out that this would be a natural consequence of their being built up of smaller structural units arranged around a rotation axis. They suggest that the most likely minimum number of such units would be 180 if all were identical, but postulate that two differing structures may be involved.

Southern bean mosaic virus (SBMV).—The studies of Magdoff (41, 42) have provided strong evidence for the existence of a subunit structure in this spherical virus. From x-ray diffraction studies of Southern bean mosaic virus crystals, this investigator has estimated the diameter of the particle to be 29.5 mμ, a value which agrees closely with the figure of 28.6 mμ obtained by Leonard *et al.* (43) from low-angle scattering of x-rays by suspensions of virus. Miller & Price have estimated that the virus has a molecular weight of 6.63×10^6 (44) and a RNA content of 21 per cent (45).

The x-ray diffraction patterns obtained by Magdoff displayed icosahedral point group symmetry, strong presumptive evidence for some sort of subunit structure. While citing the case of turnip yellow mosaic virus as an example of the hazards of interpreting such patterns, Magdoff (42) points out that if the subunits of SBMV form the protein framework of the virus with 532 symmetry, there would be 60 identical or near-identical units per particle. On the basis of a molecular weight of 6.63×10^6 (and an RNA content of 21 per cent), each such structural unit would have a molecular weight of about 87,000. The amino acid analysis of Magdoff, Block & Montie (46) suggests that each structural unit may in turn be constructed of several chemical subunits, as is the case with TYMV. From the amount of cystine present, they have calculated a minimum molecular weight of 27,000 for the protein subunit, which may mean that 3 polypeptide chains of about that size comprise each structural unit.

Other plant viruses.—Certain of the structural details of the beet yellow virus (length 1250 mμ, diameter 10 mμ) have been revealed by the electron micrographs made by Horne, Russell & Trim (47). The virus has a central hole 3 to 4 mμ in diameter, and exhibits a regular periodic structure along its axis. The images correspond best to a loose, hollow helix. The end-group analyses carried out by Niu, Shore & Knight (24) on a number of plant viruses indicate quite clearly that, in addition to TMV and BSV, potato x, cucumber 3, and cucumber 4 viruses contain repeating polypeptide units. These workers have estimated that the molecular weights of the subunits of these last three viruses are 74,000, 29,000, and 29,000, respectively, and that their C-terminal amino acids are proline, alanine, and alanine.

Animal Viruses

Information regarding the fine structure of animal viruses has been observed, with the single exception of poliovirus, through the efforts of the electron microscopists. The reason is simple. The limitations of the x-ray diffraction method are that a very large number of particles possessing a high degree of homogeneity must be examined at one time, and that these particles must be regularly arranged in space. This implies either a crystal (spherical viruses) or an oriented gel (rod-shaped viruses). Whereas a number of spherical plant viruses (bushy stunt; turnip yellow mosaic, and southern bean viruses, for example) are rather readily purified and yield well-ordered crystals, only polio among the animal viruses has fulfilled the requirements for x-ray diffraction analysis [Schaffer & Schwerdt (48); Schwerdt & Schaffer (49); Steere & Schaffer (50)].

Poliomyelitis virus.—The x-ray diffraction patterns obtained by Finch & Klug (51) showed clearly that poliovirus particles possess icosahedral symmetry, and prompted these investigators to suggest that the protein shell of the virus is built up of 60 structurally equivalent units. They found the center-to-center distance between subunits (a measure of the diameter of the unit) to be 6 to 6.5 mμ, and have calculated that if 60 protein subunits of this size were packed together in a manner consistent with icosahedral symmetry, they would form a spherical shell of 30 mμ diameter. This value is in good agreement with those previously obtained by electron microscopy [Steere & Schaffer (50); Sabin *et al.* (52); Schwerdt *et al.* (53); Schwerdt & Schaffer (54)].

Horne & Nagington (55) have applied the potassium phosphotungstate-negative staining technique to fragments of HeLa cells infected with poliovirus, and have obtained electron microscope images which bear out the predictions made by Finch & Klug. Visual confirmation of subunit structure was evident, and although the arrangement of the structural units could not be resolved unequivocally, it appeared to be consistent with the icosahedral symmetry deduced from x-ray diffraction patterns. Each of the three strains of poliovirus was found to have a dimeter of 30 mμ, and the protein subunits of each measured 6 mμ in diameter.

It is regrettable that, as yet, there has been no published chemical evidence for the presence of protein subunits in poliovirus or, for that matter, in any animal virus. The possibility that the structural subunits may be further divided into chemical units must be considered very real in the light of the information obtained from studies of the spherical plant viruses. However, for the present we must be content with an estimate of 80,000 as the maximum molecular weight of such a unit, a value calculated on the basis of a viral molecular weight of 6.8×10^6 [Schachman & Williams (56)] and an RNA content of 25 per cent [Schaffer & Schwerdt (57)].

Adenovirus.—Electron micrographs of phosphotungstate-stained type 5 adenovirus (diameter approximately 70 mμ) by Horne *et al.* (58) have revealed that the shape of this virus is icosahedral, and that it has subunit

structure. Moreover, these workers were able to determine the precise arrangement of the structural units from a comparison of the electron microscope images and a model of an icosahedron built from table tennis balls. The number of such units per virus particle is 252. Each has a diameter of about 7 mμ, and they are distributed in the following manner: 1 unit is located at each of the 12 vertices, 4 are on each of the 30 edges, and 6 are packed into each of the 20 triangular faces of the particle. Thus, three geometrically distinct units are present, for those at the vertices are surrounded by 5 other units, those on the edges by 6 others in different planes, while those on the faces are surrounded by 6 others all in the same plane. Horne and his colleagues suggest that the icosahedral shape could be obtained if three chemically different subunits were used to build the shell, or if it were determined (all subunits being identical) by the symmetry of an internal structure, which could be nucleic acid or nucleoprotein.

Herpes simplex virus.—Wildy, Russell & Horne (59) have recently obtained electron micrographs of this virus (strain HFEM) which provide a wealth of information regarding its structure. The virus consists of three main parts: the core, which is embedded in a shell (designated capsid by these investigators), and an envelope. The core, presumably consisting of nucleic acid or nucleoprotein, seems to be polyhedral in shape with an average diameter of 77.5 mμ. The capsid, icosahedral in shape with an average diameter of 105 mμ, is the most interesting component from the point of view of this survey. It is composed of 162 substructures (Wildy *et al.* have termed them capsomeres) arranged in an orderly manner showing 532 axial symmetry. These capsomers are best described as hollow, elongated, polygonal prisms with mean dimensions of 9.5×12.5 mμ and a central hole 4 mμ in diameter. These structures appear hexagonal in cross section, suggesting that each may be composed of 6 smaller subunits. Again, we must note the lack of chemical data regarding protein subunits.

SV39.—The electron micrographs of negatively stained SV39 virus (an enteric simian agent) recently published by Archetti *et al.* (60) show quite clearly that the shell of this virus is built of subunits. The virus particles appear to be polyhedral in shape, with diameters of 70 to 80 mμ. The authors estimate that the subunits have a diameter of 5 to 6 mμ, and have suggested a model in which each triangular face of an icosahedron is made up of 15 subunits, none of which occupies a position either on the edges or at the vertices of the polyhedron. Thus, the complete protein shell would contain 300 structural units.

Tumor viruses.—Convincing evidence of subunit structure has been obtained from electron microscopic examination of the polyoma virus, an agent which produces multiple tumors in hamsters and mice, and of the Shope papilloma virus of rabbits. With the latter agent, Williams, Kass & Knight (61) have shown that purified particles, after treatment with phosphotungstic acid, showed a pattern of spots over the surface and around the periphery. The spots were of uniform size and were arranged in a definite pattern, sug-

gesting that they represent subunits. Each unit is about 8 mμ in diameter, and about 30 are visible on each particle in the electron micrographs, implying that the virus may contain 60 of them.

Wildy *et al.* (62), also employing the negative contrast method, have deduced from their electron micrographs that the polyoma virus—a particle of mean diameter about 45 mμ—has in its shell (capsid) a total of 42 subunits (capsomeres). They found no evidence of icosahedral shape, but showed that the capsomere arrangement exhibited 532 axial symmetry.

Internal structure of myxoviruses.—The agents, including influenza, Newcastle disease (NDV), fowl plague and mumps viruses, which are known collectively as the myxoviruses, are more complex chemically (and perhaps physically) than any we have considered in the foregoing paragraphs. They appear roughly spherical in the electron microscope, but are much more heterogeneous, in both size and shape, than other viruses. They contain, in addition to RNA and protein, substantial quantities of lipid and polysaccharide. It has been known for many years that when exposed to ether they disintegrate to yield two particulate fractions, the nucleic acid-free hemagglutinin and a soluble antigen fraction (called G and S antigen in fowl plague and influenza, respectively), which has no lipid or polysaccharide, but which contains all the viral RNA. The soluble antigen fraction contains from 6 to 9 per cent RNA [Schäfer (63)], and until recently was always isolated in the form of fragments of molecular weight of the order of 10^6, which meant that the "molecular weight" of the RNA associated with the particles was about 60,000. Recently, however, Schäfer & Rott (64) have shown that the G antigen of fowl plague, when carefully isolated, is in the form of long, flexible and very fragile rods. This would appear to explain the earlier observations regarding the size of the soluble antigen.

Horne and his co-workers (65, 66) have now obtained electron micrographs which shed considerable light on the fine structure of the internal components of mumps, NDV, and Sendai viruses. The technique employed was the negative staining procedure which has proved so useful in defining the details of surface viral structure. No regularly arranged subunits corresponding to those seen in adeno, herpes simplex, or polio viruses could be seen on the surface of the particles. However, the structure of the inner component could be seen in those particles which had been partially disrupted.

The long, fibrous material released from mumps virus was found to have a diameter of 17 mμ, and a regular periodic structure along the axis. Small lengths, suitably oriented, showed a central hole with a diameter of 4 mμ. The inner component of the Sendai virus was found to have roughly the same dimensions as that of the mumps virus, and was characterized by the same periodicity (of 5 mμ) along the axis. The obvious explanation for this periodicity is that it is the result of a helical arrangement within the strand. Horne & Waterson (65) made the additional, very exciting observation that in the case of the Sendai virus, the structure may have a double helical array, and pointed out that the existence of such a structure with separate RNA

chains might provide a basis for an explanation of heterozygosis and diploidy in myxoviruses [Gotlieb & Hirst (67); Granoff & Hirst (68)]. Particles have also been observed in disrupted particles of NDV. Their dimensions are similar to those seen with mumps and Sendai viruses, but the arrangement within the tubular structure may differ in that the helix appears to be more tightly packed. Horne & Waterson suggest the possibility that the subunits of neighboring strands may interlock with one another.

Horne *et al.* (66) have reported that although a tightly packed inner structure could be seen in influenza and fowl plague viruses stained with phosphotungstate, the details of this component could not be determined because so few disrupted particles were encountered. They do comment, however, that a helical inner component which may be double stranded has been demonstrated in ether-disrupted virus. They promise a full description of these findings in the near future.

Avian Viruses

GAL (gallus, adeno-like) virus.—Reports on the fine structure of this agent, which is similar in several respects to the human adenoviruses [Burmester *et al.* (69)], have been presented by two groups of investigators, Davies & Englert (70) and Macpherson and co-workers (71). The results obtained by the two groups leave no doubt as to the subunit construction of the protein shell, or that the virus presents a hexagonal profile. However, though both groups agree that the particle is, in all probability, an icosahedron, they differ as to the details of the structure. Davies & Englert quote an average particle diameter of 86 mμ and a subunit diameter of 9.5 mμ, while Macpherson *et al.* report values of 97.5 mμ and 5 to 6 mμ for these two parameters. Davies & Englert interpret their electron microscope images as indicating that the virus shell consists of 162 structural units. Although they do not spell out as clearly as they might the proposed distribution of these units, we interpret their comments to mean that 12 units occupy the 12 vertices, that each of the 30 edges hold 3 additional units and that the remaining 60 are distributed 3 to each of the 20 triangular faces of the polyhedron. Macpherson *et al.*, on the other hand, feel that the protein shell consists of 252 subunits, distributed in the same manner as that suggested by Horne *et al.* (58) for the adenovirus. The electron micrographs obtained by Macpherson *et al.* indicate an additional structural detail. The subunits (capsomeres) appear to be hollow, elongated prisms 10 to 11 mμ long and 5 to 6 mμ wide with an axial hole 2 to 2.5 mμ in diameter, suggesting, of course, the possible existence of chemical subunits smaller than the structural units seen in the electron micrographs.

Bacterial Viruses

Bacteriophage T2.—It is probably safe to say that no single virus has been the subject of such extensive investigation, or of so many reviews, as has the T2 coliphage. It is not our intention to present yet another review,

even in capsule form. We refer the reader to those of Hershey (72), Tolmach (73), and Garen & Kozloff (74) for a comprehensive survey of phage morphology and composition, and of the mechanism of attachment and penetration. Cohen (75) has recently summarized the information available regarding the effects of T2 infection on the metabolism of the host (*E. coli*) cell. However, the recent studies of Brenner and co-workers, which have provided new information on the fine structure of several of the morphological components of this virus, fit logically into this section.

Brenner *et al.* (76) have described the isolation of tail sheaths and fibers from the T-even bacteriophages, and their characterization by electron microscopy as well as by physical and chemical means. Electron micrographs of purified sheaths (embedded in phosphotungstic acid) revealed that in their contracted form they are hollow cylinders 35 mμ long and 25 mμ in diameter, the diameter of the inner hole being 12 mμ. In extended form the sheath has a diameter of 16.5 mμ and a length of 80 mμ. Structures standing upright were found to display a "cog wheel" appearance, while those lying obliquely showed helical grooving. This suggests that the sheath is constructed of subunits and that their arrangement is helical. The fact that striations (about 25 in number with a spacing of 3 to 4 mμ) are seen in negatively stained extended sheaths reinforces this picture.

On the basis of complete amino acid analyses coupled with data obtained from fingerprinting tryptic digests by the method devised by Ingram (77), Brenner *et al.* estimated that the chemical subunit of the sheath has a molecular weight of about 50,000, and, by combining this value with the particle weight of the intact sheath, calculated that each sheath would contain some 200 subunits. The electron micrographs indicated that one turn of the contracted sheath contains 15 subunits. On the basis of 200 units per sheath, this means that the entire sheath would contain about 13 turns. The number of striations seen in the extended sheath is about 25, suggesting that there are 25 turns of the helix in the extended structure. All of these values become consistent in the light of the observed contraction of sheaths to about one-half their extended length.

Tail fibers were found to be long, slender structures, 130×2 mμ, bent in the middle. The authors suggest that the length of the fiber may be determined by the length of a single polypeptide chain, and that the fiber may be a bundle of such chains. They point out that an α-helix 130 mμ long would contain about 860 amino acids and have a molecular weight of about 100,000, of value very close to that estimated from fingerprints of tryptic digests for the chemical subunit of the tail fiber. From the dimensions observed in the electron micrographs, the particle weight of the entire fiber was estimated to be of the order of 400,000.

Bacteriophage ϕX174.—Hall *et al.* (78) have examined the small (diameter approximately 25 mμ), spherical bacteriophage ϕX174, employing the methods of shadowcasting with platinum, and negative staining. In micrographs of the virus prepared by the latter method, the agent appeared poly-

hedral in outline. Shadowcast preparations showed the presence of what the authors call "knobs" on the surface of the phage particle. They interpret their pictures as indicating that the virus contains a shell of 12 structural units arranged as on the face of a regular dodecahedron. On the basis of a particle weight of 6.2×10^6 and a DNA content of 25 per cent [Sinsheimer (79, 80)], this would mean that the "molecular weight" of each structural unit would be about 400,000. Assuming that the phage DNA contains the code for the synthesis of its own protein, and assuming further a triplet code, the maximum molecular weight of the protein would be of the order of 200,000. It seems altogether likely that the structural units observed in electron micrographs are themselves constructed of a number of chemical subunits.

Insect Viruses

Tipula *iridescent virus* (*TIV*).—The shape of this large (diameter 130 mμ) cytoplasmic virus of the dipterous insect *Tipula paludosa* has been determined most elegantly by Williams & Smith (81). These workers first showed that in frozen-dried, unshadowed preparations, the virus presented an almost perfect hexagonal profile, from which they deduced that the virus is icosahedral in shape. Convincing evidence that this is so was provided by shadow analysis. The virus particles were shadowed in one or two directions and the shapes of the shadows were recorded in electron micrographs. These were then compared with the shadows cast by a cardboard model of an icosahedron set in various orientations in a beam of white light, and with the shadows observed when the model was set in the paths of two light sources. The data leave no doubt as to the icosahedral shape of the virus. Smith & Hills (82) have examined the *Tipula* iridescent virus by the negative staining method, and have observed the presence of subunits on the icosahedral faces of the particle. The electron microscope images could not be interpreted to provide information regarding either the number of subunits comprising the shell or their packing arrangement.

INFECTIOUS VIRAL NUCLEIC ACIDS

Although the unique importance of bacteriophage DNA in the replication and genetic continuity of these viruses had been recognized since 1952 [Hershey & Chase (83)], it was not until 1956 that the corresponding central role played by viral RNA was directly demonstrated by Gierer & Schramm (84). These investigators showed that RNA isolated from TMV was capable of initiating an infection in tobacco leaves leading to the formation of virus particles identical to those from which the nucleic acid was isolated. During the past few years the work of Gierer & Schramm has been extended by many investigators, until now the list of mammalian and plant viruses which have yielded infectious RNA is a rather imposing one. These viruses are listed in Tables I and II.

TABLE I

INFECTIOUS RIBONUCLEIC ACIDS FROM PLANT VIRUSES

Virus	Extraction method	Plant used for assay	Investigators
Tobacco mosaic virus	Phenol	Tobacco	Gierer & Schramm (84)
Tobacco mosaic virus	Detergent	Tobacco	Fraenkel-Conrat *et al.* (85)
Necrotic ringspot virus	Phenol	Cucumber	Diener & Weaver (86)
Tobacco rattle virus	Phenol	French bean	Harrison & Nixon (87)
Tobacco ringspot virus	Heat denaturation	Black-eye cowpea	Kaper & Steere (88)
Turnip yellow mosaic virus	Heat denaturation	Chinese cabbage	Kaper & Steere (89)
Potato virus x	Guanidine·HCl	*Chenopodium amaranticolor*	Reichmann & Stace-Smith (90)
Tomato bushy stunt virus	Detergent and phenol	Tobacco	Rushizky & Knight (91)
Cucumber mosaic virus	Phenol	Cowpea	Schlegel (92)
	Phenol	Black cowpea	Welkie (93)

Isolation of viral RNA.—The technique described by Gierer & Schramm (84) for the isolation of RNA, the so-called phenol method, has been used almost exclusively for the preparation of infectious RNA from mammalian viruses. Anderson & Ada (110) have, however, shown that incubation of Murray Valley encephalitis virus with 1 per cent deoxycholate results in the formation of an infectious product which, like that liberated by extraction of the agent with water-saturated phenol, is completely sensitive to ribonuclease (RNase). The infectivities of the products formed by the action of the two agents were found to be essentially the same.

Plant viruses, on the other hand, have succumbed to a number of extraction procedures in addition to the phenol method. Infectious RNA has been obtained from TMV by both phenol and detergent (sodium dodecyl sulfate) treatment. Kaper & Steere (88, 89) have employed a heat denaturation method to release biologically active RNA from turnip yellow mosaic and tobacco ringspot viruses. Reichmann & Stace-Smith (90) showed that while extraction of potato virus x with phenol failed to release infectious RNA, incubation of the agent with 2.5 M guanidine·HCl did. Bawden & Kleczkowski (118) had previously reported their failure to obtain active RNA from this virus by the phenol method. Rushizky & Knight (91) found that a combination of detergent and water-saturated phenol gave biologically active RNA from tomato bushy stunt virus.

Activity of influenza virus RNA.—Conspicuous by their absence from Table II are the several reports concerning the isolation of infectious RNA from influenza virus. They have been omitted because we feel that the situation with respect to this virus is crying out for clarification. However, the reports merit some discussion. Maassab (119) has reported the isolation, from influenza virus-infected chorioallantoic membranes, of RNA which was infectious in chick kidney cultures but not when injected into the allantoic cavity of the embryonated egg. It might be recalled that Wecker and his colleague, Schäfer (103, 104) found the embryonated egg to be a

sensitive system for measuring the infectivity of RNA isolated from the Eastern and Western equine encephalitides. Maassab concluded that his RNA preparations were infectious on the basis of cytopathology produced in exposed cultures, and of a positive hemadsorption reaction therein. The progeny resulting from infection with RNA grew in chick kidney cultures to a much lower titer than did the original virus, and, unlike the latter, did not replicate at all in the allantoic cavity of the embryonated egg. No account was given of a serological characterization of the "progeny virus," which leaves room for doubt as to its identity. The observation that the progeny virus had a different host range than did the parent virus, has not been made with other viruses yielding infectious RNA. If this could be rigorously confirmed, it would provide an example of either a host-induced modification, or a selection of a variant genome from the infecting viral RNA. Maassab

TABLE II

INFECTIOUS RNA FROM MAMMALIAN VIRUSES

Virus	Assay system	Investigators
Mengo encephalomyelitis	Mice (intracerebral)	Colter *et al.* (94, 95)
	Tissue culture ("L" cells)	Ellem & Colter (96, 97)
West Nile encephalitis	Mice (intracerebral)	Colter *et al.* (95)
Poliomyelitis	Mice (intracerebral)	Colter *et al.* (95)
	Tissue Culture (HeLa cells)	Alexander *et al.* (98)
	Tissue Culture (HeLa cells)	Schaffer & Mattern (99)
	Tissue Culture (HeLa, monkey kidney cells)	Holland *et al.* (100, 101)
Semliki forest encephalitis	Mice (intracerebral)	Cheng (102)
Eastern equine encephalitis	Embryonated eggs (allantoic cavity)	Wecker & Schäfer (103)
Western equine encephalitis	Embryonated egg (allantoic cavity), chick embryo fibroblast cells	Wecker (104, 105)
Mouse encephalomyelitis virus	Mice (intracerebral)	Franklin *et. al.* (106)
Encephalomyocarditis virus	Krebs 2 ascites tumor cells	Huppert & Sanders (107)
	Mice (intracerebral)	Liebenow & Schmidt (108)
Murray Valley encephalitis	Chick embryos (chorioallantoic membrane)	Ada & Anderson (109)
	Chick embryos (chorioallantoic membrane)	Anderson & Ada (110)
Dengue I and II	Mice (intracerebral)	Ada & Anderson (111)
Theilers GD VII	Mice (intracerebral)	Ada & Anderson (111)
Foot-and-mouth disease virus	Pig kidney cell cultures, mice (intramuscular)	Brown *et al.* (112)
	Bovine kidney cell cultures	Bachrach (113)
	Mice (intracerebral)	Mussgay *et al.* (114)
	Guinea pig (route of administration not given)	Spuhler (115)
Tick-borne encephalitis (strain Hypr)	Mice (intracerebral)	Sokol *et al.* (116)
Coxsackie A-7, B-4, B-5	HeLa and human amnion cell cultures	Sprunt *et al.* (117)
Coxsackie A-9, B-1	Tissue culture (HeLa, human amnion and others)	Holland *et al.* (101)
ECHO 1 and 8	HeLa and human amnion cell cultures	Sprunt *et al.* (117)
ECHO 8	Tissue culture (HeLa, human amnion and others)	Holland *et al.* (101)

was unable to isolate active RNA from purified suspensions of the virus, from which he concluded that his active material was derived from some virus precursor.

Portocalà *et al.* (120, 121, 122) have reported the isolation of RNA from purified influenza virus which was infectious when injected into the allantoic cavity of embryonated eggs. They stated that a preliminary extraction of the virus with ether was necessary for the successful release of active RNA by phenol extraction. In two of their publications (120, 121) these investigators reported that the progeny resulting from an RNA-initiated infection had a somewhat different antigenic composition than did the preparation from which the nucleic acid was isolated, and, that after a second passage in the embryonated egg, the new virus had a filamentous form, a characteristic not seen in the original virus. Two possible mechanisms which might explain this lack of identity between progeny and parent viruses were discussed in relation to Maassab's results. Nonetheless, the observations are hard to reconcile with the observations of other investigators that the biological properties of a virus are retained by the progeny resulting from an infection produced by RNA from that virus. Gerber & Kirschstein (123), for example, have shown that the temperature markers of virulent and attenuated type 3 polioviruses are retained after passage of the strains by means of their respective ribonucleic acids. Portocalà and his co-workers (120) have also reported that, in one of two attempts, they were able to isolate infectious RNA from Newcastle Disease virus. However, no experimental details were given.

On the other side of the ledger are a number of papers dealing with the failure of attempts to isolate biologically active RNA from myxoviruses. Schäfer (124) has reported that he was unable to isolate active nucleic acid from fowl plague virus. Ada *et al.* (125) have issued a similar report on NDV and several strains of influenza virus. Sokol & Szurman (126) were unable to isolate infectious RNA from influenza virus even though they utilized the isolation procedure of Portocalà *et al.* Benedict *et al.* (127) have reported on their extensive efforts to extract biologically active RNA from NDV. Although they employed a number of extraction procedures (hot and cold phenol, hot 1 *M* sodium chloride, 8 *M* urea, 2.5 *M* guanidine), utilized purified virus and infected allantoic membranes and fluids as potential sources of RNA, and tested the preparations in the embryonated egg and in chick embryo fibroblast cultures, their results were uniformly negative. The reports of Maassab and of Portocalà *et al.* are suggestive but far from conclusive.

Assay systems for viral RNA.—Whereas the appropriate intact plant has been used as the test system for each of the plant virus RNA preparations, both *in vivo* and *in vitro* systems have been used to measure the infectivity of RNA isolated from mammalian viruses. The activity of infectious RNA is most readily (and most precisely) estimated in an *in vitro* system, assuming that a suitable one can be devised. Extracellular RNase can be removed by

washing, the conditions under which cells and RNA interact can be carefully controlled, and the results can be quantitated readily.

Two different plaque methods have been employed for the assay of infectious viral RNA. In one, the RNA solution is pipetted directly onto well-washed monolayers of susceptible cells. After a brief incubation period, the monolayers are covered with an agar overlay. After several days the monolayers are stained with a vital dye and the plaques are counted. The second method involves incubating washed cells (as a suspension of single cells) in the RNA solution under investigation, followed by an examination of the treated cells for the presence of infectious centers. In these laboratories, where the latter method has been developed [Ellem & Colter (96)], the procedure used is described below.

A known number of cells (usually 10^6 or 2×10^6) is suspended in 1 ml. of viral RNA solution and incubated in a water bath for a precise length of time, after which the suspension is diluted to 10 ml. with tissue culture growth medium ($2\times$Eagle's in Earle's solution containing 10 per cent horse serum). Aliquots of the suspension of treated cells, or of further dilutions thereof, are pipetted onto preformed monolayers of susceptible cells. After an incubation period of 1 to 1.5 hr., during which the test cells settle out and adhere to the monolayer, the medium is removed and replaced with an agar overlay. Plaques are visualized and read in the usual manner after 2 or 3 days.

The suspended cell system has certain advantages over that in which the RNA is titrated directly onto monolayers. First, under the conditions of the suspended cell system, the statistics of discrete distributions can readily be applied, and the cell population can be exposed uniformly to modifications of the environment in a precisely quantitated fashion. Secondly, the efficiency with which RNA infects cells is markedly increased when the nucleic acid is dissolved in solutions of elevated ionic strength, an observation first made by Alexander *et al.* (98) and later confirmed by Holland and his coworkers (101). However, monolayers of some cell types are damaged by exposure to hypertonic solutions ('L' cells, for example, are damaged by even brief exposure to hypertonic NaCl solutions), and the resulting cell degeneration can result in areas of poor plaque definition. This difficulty is circumvented by use of the suspended cell system. Finally, this system has made it possible to define the optimal conditions for cell-RNA interaction, and to investigate with some precision the kinetics of the interaction. One characteristic of the system should be emphasized regarding the latter point. Dilution of the cell-RNA mixture with tissue culture growth medium immediately and completely abolishes any further cell-RNA interaction. There is no carry-over of infectious RNA units to the indicator monolayer other than those which have become stabilized by interaction with cells.

Optimal conditions for cell-RNA interaction.—Alexander *et al.* (98) found that they obtained maximum efficiency of infection of HeLa cell monolayers with poliovirus RNA when the RNA solution was made 1 M with respect to

sodium chloride. Holland *et al.* (128) have assayed enteroviral RNA preparations by pipetting 0.2 ml. volumes in buffered 2 *M* magnesium sulfate onto HeLa cell monolayers. In both cases, the extracellular fluid would be rapidly diluted due to dehydration of the cells, so that one could only approximate its final ionic strength and concentration of RNA.

Ellem & Colter (96) have examined the effect of osmolarity on the formation of infectious centers in Earle's 'L' cell-Mengo virus RNA mixtures. Since the volume of the test cells is extremely small compared to that of the RNA solution, the ionic strength of the latter is not significantly altered by dehydration of the cells. Incubations were carried out in phosphate-buffered sodium chloride solutions and in solutions of sucrose in phosphate-buffered physiological saline (*x M* sucrose-PBS). A sharp optimum was found in each series. Maximum formation of infectious centers was seen at sodium chloride and sucrose concentrations of 0.64 *M* and 0.7 *M*, respectively. The number of infectious centers produced in the optimal sucrose solution was 1.5 to 2.0 times the number produced in the optimal sodium chloride solution. The osmotic pressures of the two media are identical, being 4.1 times that of physiological saline.

Similar investigations of the HeLa cell-poliovirus RNA system yielded quite different results [Ellem & Colter (unpublished data)]. Although sharp optima were again observed in the curves relating the number of infectious centers to the solute concentration of the media in which cells and RNA were incubated, the optimal sodium chloride and sucrose concentrations were found to be 0.90 *M* and 0.98 *M*, respectively. The number of infectious centers produced in the optimal sodium chloride solution exceeded the number formed in the optimal sucrose solution by a factor of 5 to 6. In this system also, the osmotic pressures of the two optimal solutions were the same. It seems evident that the conditions which are optimal for the infection of cells with viral RNA depend upon the particular cell employed.

The question of the mechanism whereby solutions of high osmolarity stimulate the establishment of infectious centers has not been completely resolved. It is clear, however, that the effect of such solutions is on the cell, not on the RNA. At 37°C., RNA is completely soluble in all sodium chloride and sucrose solutions investigated. The rate of thermal inactivation of Mengo RNA has been shown [Colter & Ellem (129)] to be the same in 0.14 *M* NaCl, 0.64 *M* NaCl, and 0.7 *M* sucrose-PBS, and the amount of RNA absorbed by the cells is essentially the same in these three media. In hypertonic solution, the cells will be dehydrated rapidly, creating a hypertonic intracellular environment This might inhibit the activity of any intracellular RNase. Crystalline RNase has been shown to be inhibited by solutions of high ionic strength [Kalnitsky *et al.* (130)].

Another possible mechanism is suggested by the observation, made in these laboratories, that the activity of Mengo RNA is abolished by several RNase-free proteins when nucleic acid and protein are mixed in solutions of physiological (but not elevated) ionic strength [Brown *et al.* (131)]. It is

possible that a hypertonic intracellular environment could prevent the non-specific immobilization of viral RNA by cytoplasmic protein.

Kinetics of cell-RNA interaction.—Employing the suspended cell system previously described, and the 0.7 *M* sucrose-PBS medium which had been found to be optimal for 'L' cell-RNA interaction, Ellem & Colter (97) have investigated the kinetics of the interaction between these cells and RNA isolated from Mengo encephalomyelitis virus. Their pertinent findings are enumerated as follows: (*a*) The number of infectious centers produced in a fixed number of cells is directly proportional to the concentration of RNA in the solution in which the cells are incubated—but independent of the volume of the solution. The same number of infectious centers are formed when 10^6 cells are incubated in 0.5, 1.0, and 2.0 ml. aliquots of the same RNA solution. (*b*) The number of infectious centers formed in a cell-RNA mixture is directly proportional to the number of cells used. As many as 20×10^6 cells do not remove all the infectious RNA from 1 ml. of solution. (*c*) The rate of formation of infectious centers in 'L' cell-Mengo RNA mixtures is markedly temperature-dependent. At 37°, 27°, and 17°C., the maximum number of infectious centers is formed in 15, 40, and 60 min., respectively. In each case, the rate is linear up to the point of maximum infectious center formation. If incubation is continued past that point there is a rapid decrease in the number of infectious centers present. The rate at 27°C. is about one-fifth that at 37°. Similar large reductions in the rate occur at 17° and 7°C. The effect of temperature on the reaction rate can be expressed in terms of the Arrhenius equation [Arrhenius (132)] for irreversible first-order reactions. The temperature characteristic (a measure of activation energy) of the system is high, 18,700 cal./mole and 33,900 cal./mole for the temperature ranges 25° to 40° and 6° to 21°C., respectively.

The kinetics of the interaction of HeLa cells and poliovirus RNA in the suspended cell system differ only in detail from those of the system just considered. The rate at which infectious centers are formed in the optimal sodium chloride solution is rapid and linear with respect to time for 8 min. (the optimal incubation period at 37°C. for the 'L' cell-Mengo RNA system is 15 min.), after which there is a precipitous drop in the number of infectious centers present.

From a consideration of the kinetics of cell-viral RNA interaction, it appears that the uptake of RNA may be a case of concentration-dependent diffusion across the cell membrane. It follows that existing methods of assaying viral RNA preparations provide more a measure of the early rate of reaction between cells and RNA than of the absolute infectivity of the nucleic acid. We suggest that viral RNA solutions, when carefully prepared, may contain as many potentially infectious units as do the corresponding viral suspensions, and that the failure to detect them reflects merely the inadequacies of the assay methods. Indeed, studies of the uptake of P^{32}-labeled RNA carried out by Holland *et al.* (128) and by Ellem & Colter (unpublished data) tend to bear out this premise. Both groups found that the amount

of RNA taken up by cells under assay conditions is a small fraction (0.1 to 0.2 per cent) of the total RNA in the mixture. If one is willing to assume that P^{32} and infectious RNA uptake can be equated, this provides an explanation for the low infectivity of viral RNA preparations reported by all investigators in this field.

The P^{32}-RNA uptake studies conducted by Ellem & Colter also showed that at 37°C., uptake was linear for at least 60 min. This observation underscores a question raised by the kinetic studies: what is the reason for the sharp break in the curve relating formation of infectious centers and time of incubation? A natural assumption is that the rapid decrease in the number of infectious centers after 8 and 15 min. (in the HeLa and 'L' cell systems, respectively) reflects a loss of viability of the cells. However, it has been found in these laboratories that cell viability, as estimated by the gross criteria of trypan blue permeability or neutral red concentrating ability, is not lost at the time when the sharp decline in the number of infectious centers is observed. Preliminary data, using a more sensitive (and appropriate) measure of cell function, namely, the ability of virus-infected cells to support viral replication after incubation in solutions of high osmolarity, indicate that the loss of this capability parallels the decrease in infectious centers seen in the suspended cell RNA-assay system.

Sources of infectious viral RNA.—A number of investigators have attempted to ascertain whether the infectious RNA obtained by extracting virus-infected cells or tissues with phenol comes from intact virus particles or from some virus precursor, perhaps viral RNA which has not been incorporated into complete virus particles. It is now quite clear that, depending upon the particular virus under investigation and the method of isolation employed, it can come from either source.

Colter *et al.* (95) incubated homogenates of Mengo-infected Ehrlich ascites tumor cells with RNase and found that they could subsequently isolate infectious RNA. On the assumption that any RNA-containing, subviral particle would be degraded by the enzyme, they concluded that at least a part of the infectious RNA isolated from the virus-infected tissue came from intact virus particles. Franklin *et al.* (106) have shown that biologically active RNA can be extracted by cold phenol from highly purified mouse encephalomyelitis virus. It is abundantly clear from the studies reported by Alexander *et al.* (98), Holland *et al.* (128), and by Schaffer & Mattern (99) that infectious RNA can be obtained from highly purified poliovirus by extraction with cold phenol. This, of course, does not rule out the possibility that when poliovirus-infected cells or tissues are extracted, some of the infectious RNA is obtained from viral precursor RNA.

The most elegant studies relating to this question have been those carried out on Western and Eastern equine encephalitis viruses (WEE and EEE). Wecker & Schäfer (103) found that extraction of WEE- and EEE-infected cells with cold phenol (4°C.) yielded an infectious RNA, while cold phenol extraction of the purified viruses did not. They reported further that when

infected cells were treated with RNase prior to extraction with cold phenol, no infectious RNA was obtained. Since the intact viruses are not vulnerable to the action of RNase, it was concluded that the active RNA obtained by cold phenol extraction came from a virus precursor (probably viral RNA itself). The picture was completed when Wecker (105) demonstrated that infectious RNA could be isolated from highly purified WEE by phenol extraction at 50°C. He suggested that only at elevated temperatures could phenol split the protective protein-lipid shell of the virus and so release the RNA moiety. At any rate, in the case of the viruses of the equine encephalitides, extraction with hot phenol will yield infectious RNA from both intact viruses and their precursors, while extraction with cold phenol will yield infectious RNA from the precursors only.

Huppert & Sanders (107) and Sanders (133) feel that the infectious RNA isolated from Krebs-2 ascites tumor cells infected with encephalomyocarditis (EMC) virus by cold phenol extraction arises from a virus precursor. They have further suggested that the virus precursor, which they have termed virosome, is resistant to RNase. These workers based their conclusions in large measure on the observation that when EMC virus-infected cell homogenates were centrifuged (60 min., 100,000g), infectious RNA could be isolated by cold phenol from the supernatant but not from the virus pellet. Similar investigations have been carried out in these laboratories employing EMC virus-infected 'L' cell cultures. Cold phenol extractions performed in the manner prescribed by Gierer & Schramm (84) yielded infectious RNA from both supernatants and virus pellets. Moreover, the amounts of infectious RNA obtained from the fractions was directly proportional to the number of virus particles therein. The difference in the findings of the two groups is puzzling. They may be explained by the fact that different systems were used, though this implies that infection of Krebs-2 cells with EMC virus results in the production of sizeable quantities of RNase-insensitive precursor, whereas infection of 'L' cells with this agent does not. It is hoped that future work will clarify the picture.

Host range of infectious viral RNA.—One of the most exciting observations that has been made with infectious viral ribonucleic acids is that they can, in a number of cases at least, infect cells which are insusceptible to infection with the corresponding viruses. Holland *et al.* (100, 101) have demonstrated that RNA preparations, isolated from polio, Coxsackie A-9, Coxsackie B-1, and ECHO 8 viruses, are capable of infecting a number of non-primate cells (from rabbit, swine, mouse, guinea pig, chicken, and hamster), which are not vulnerable to the intact agents themselves. In these laboratories it has been found that HeLa cells, which are resistant to Mengo encephalomyelitis virus, can be infected with RNA from this agent.

The progeny resulting from such infectious processes appear to be identical to the parent viruses. No evidence has been obtained which would suggest an altered host range, or indicate a one-step adaptation to the normally insusceptible host. Infection with viral RNA of cells insusceptible to the

intact virus results in a single cycle of replication only. Virus particles released from the infected cells do not infect other cells in the same culture, and for this reason detectable cytopathology is not seen. Production of virus by "insusceptible cells" may be demonstrated by titrating the released virus in cultures of susceptible cells. Holland *et al.* (134) have also trypsinized cultures of "insusceptible cells," after exposure to RNA, but before release of virus, and have estimated the number of infectious centers in the resulting cell suspension by titration on monolayers of susceptible cells.

These investigations have strengthened a widely held concept for which direct experimental evidence was lacking, namely that the extranucleic acid portion of mammalian viruses does not function merely as a protective coat for the nucleic acid, but plays a central role in determining the host range (trophism) of the agents by providing specific mechanisms for their efficient attachment to and penetration of host cells.

Physicochemical studies of viral RNA.—Fairly extensive physicochemical studies have been carried out on TMV-RNA. Comparable studies on infectious RNA from mammalian viruses have not been done, presumably because of the same technical problems which have so severely limited x-ray diffraction studies of mammalian viruses. Simply stated, it is extremely difficult to get sufficient material to work with, and, in most cases, even more difficult to be sure that the material one does obtain consists of viral RNA only.

In the case of TMV-RNA, it is now quite clear that the infectious material consists of the complete nucleic acid complement of the virus particle, the uninterrupted sequence of 6000 plus nucleotides. Gierer (135, 136, 137) has carried out coordinated studies of the physical and biological properties of RNA isolated from TMV by the phenol method. He indicated that the molecule has a weight of 2×10^6, and that its biological activity is completely dependent on its integrity. From studies correlating biological activity, sedimentation velocity, and intrinsic viscosity, he concluded that a single break in the polynucleotide chain results in the loss of infectivity. Boedtker (138) has characterized infectious TMV-RNA, prepared by a heat denaturation procedure, by light-scattering and sedimentation-viscosity measurements. She concluded that the active material had a molecular weight of $1.94\pm0.16\times10^6$, and showed that the loss of biological activity on standing resulted in a broadening of the distribution of sedimentation constants and a decay of molecular weight. Sinsheimer and his colleagues (139, 140) have applied the techniques of light-scattering and sedimentation velocity to both phenol- and detergent-prepared TMV-RNA. They concluded that the molecular weight of the infectious component was 2×10^6, regardless of the method of isolation of the nucleic acid. The molecular weight of the total RNA content of a single TMV particle, calculated from a particle weight of 40×10^6 and a RNA content of 5 to 6 per cent is in excellent agreement with the results of these investigators.

Although data regarding the size of RNA from mammalian viruses are

neither extensive nor definitive, they do indicate that the biologically active nucleic acid molecules correspond to the total RNA of the virus particles and have molecular weights of the order of 2×10^6. Colter *et al.* (95) showed that RNA isolated from virus-infected tissues exhibited two components in the analytical ultracentrifuge with sedimentation velocities of 16S and 32S, and that the infectious components seemed to travel with the faster sedimenting component. This observation, coupled with the light-scattering studies of phenol-prepared RNA by Timasheff *et al.* (141) prompted the suggestion that the molecular weight of RNA from Mengo, West Nile, and polio viruses is of the order of 1.5 to 2.0×10^6 [Colter (142)]. Poliovirus has been found to have a particle weight of 6.8×10^6 and an RNA content of 25 to 30 per cent, from which the molecular weight of RNA from a single virus particle may be calculated to be 1.7 to 2.04×10^6. No satisfactory chemical data on Mengo and West Nile viruses are available. Wecker (143), on the basis of ultracentrifugation, has estimated the molecular weight of EEE-RNA to be about 2×10^6. Strohmaier & Mussgay (144, 145) have examined the RNA of foot-and-mouth disease virus by density gradient centrifugation and have suggested that it has a molecular weight of 3.1×10^6.

Infectious viral DNA.—In 1957, Spizizen (146) and Fraser *et al.* (147) reported that suspensions of T2 bacteriophage, disrupted by osmotic shock or by concentrated urea, were capable of infecting *E. coli* protoplasts; and that complete T2 phage particles, fully infectious to intact *E. coli* cells, were produced. These workers were able to show that the infectious particles in their preparations were not undamaged bacteriophage particles, but not that they were protein-free T2-DNA molecules. In fact, they suggested that the infectious component was phage DNA with some phage protein, essential for activity, still attached. Their conclusion has been verified by Sekiguchi (148) who showed that the biological activity of urea-disrupted T2 bacteriophage was destroyed more rapidly by proteolytic enzymes than by DNase, and that $CHCl_3$ purification also destroyed infectivity. Both groups made the exciting observation that their preparations were capable of infecting protoplasts derived from certain strains of bacteria resistant to the intact virus, an observation reminiscent of the more recent studies of Holland and co-workers on the infection of "insusceptible" mammalian cells with viral RNA.

One of us [Colter (149)], in a review written at about that time, predicted that unequivocal evidence in support of the premise that protein-free T2-DNA could be infectious, would soon be forthcoming, thus demonstrating unequivocally that this reviewer is something less than a seer. However, it has recently been shown that DNA preparations isolated from several other viruses are infectious, and these reports are discussed in what follows.

The first such report was that of Di Mayorca *et al.* (150), who showed that infectious DNA could be isolated from the polyoma virus. Two methods (both successful) were employed to prepare the nucleic acid from virus suspensions: the method described by Gierer & Schramm for the isolation of

RNA, and that of Kirby (151), which also involves the removal of protein by phenol denaturation. The identity of the active component was established enzymatically. It was destroyed by DNase but not by RNase. The infectivity of the nucleic acid was demonstrated in primary cultures of mouse embryo fibroblasts. Reproducible cytopathogenic effects were obtained, and the production of intact virus in the cultures was shown by the ability of the culture medium to induce cytopathogenic changes when passaged further in mouse embryo fibroblast cultures, to hemagglutinate, and to produce tumors when injected into newborn hamsters.

More recently, Weil (152) has described a more precise method for the assay of phenol-prepared polyoma virus DNA. The method, an adaptation of that previously developed for polyoma virus [Dulbecco & Freeman (153); Winocour & Sachs (154)], depends upon the formation of plaques in monolayers of mouse embryo fibroblasts. Some of the conditions which affect plaque formation by polyoma DNA were described. The efficiency of infection was increased by washing the monolayers (preferably with hypertonic solutions) prior to applying the DNA solution. The ionic strength of the DNA solution was found to be a most important factor in determining the number of plaques formed. Versene was found to have a marked stabilizing effect on the active nucleic acid. Plaque formation was shown to be strongly temperature-dependent, and the period of incubation required to give maximum plaque-formation increased progressively as the incubation temperature was decreased from 34° to 29° to 24°C. The similarity of the factors which affect plaque formation in this system and in systems involving viral RNA is quite striking. Plaques formed as a result of infection with polyoma DNA exhibited the same features as those developed by the virus from which the nucleic acid was isolated. DNA preparations from large and small plaque variants of the virus produced large and small plaques, respectively.

Guthrie & Sinsheimer (155) have demonstrated conclusively that DNA isolated from bacteriophage $\phi \times 174$ can infect protoplasts made from a number of strains of *E. coli*, some of which are completely resistant to infection with the intact virus. Infectious DNA was isolated by three different methods: heat denaturation, phenol extraction, and a method involving the precipitation and incubation of the virus in cold 4 M $CaCl_2$ followed by the removal of the viral protein by resuspending the virus pellet in Tris buffer, pH 7.5 to 8.0. Phenol- and calcium-prepared DNA were judged to be identical on the basis of sedimentation velocity measurements and banding by cesium chloride density gradient centrifugation. The infectious DNA preparations were destroyed by DNase, but were not inactivated by enzymatic amounts of trypsin. From preliminary kinetic studies, the authors concluded that with this DNA (as with TMV and TMV-RNA) there is no specific attachment mechanism by which efficient infection can take place.

Sekiguchi and his co-workers (156) and Wahl *et al.* (157) have also reported the successful isolation of infectious DNA from bacteriophage $\phi \times 174$. The first group isolated DNA by heating the virus suspension (90°C. for 10

min.), and then, after making the solution 1 *M* with respect to NaCl, deproteinizing the preparation by shaking with chloroform-octanol (8:1); Wahl and co-workers used both dodecylsulfate and phenol to prepare active nucleic acid solutions. Both groups found that their preparations were rapidly inactivated by DNase, but not by trypsin or RNase; and the Japanese workers demonstrated, as did Guthrie & Sinsheimer, that the ϕ X-DNA was capable of infecting protoplasts made from bacteria resistant to the intact virus.

One final notation must be added. Ito (158) has reported that DNA, isolated from Shope papilloma of wild cottontail rabbits by either hot or cold phenol, produced papillomas when injected into the skin of domestic rabbits. The phenomenon was reproducible and, when the DNA was injected by the "injection and puncture" method, the ratio of positive growth:number of inoculation sites was close to unity. The activity of his preparations was destroyed by DNase but not by RNase. Antisera against Shope papilloma virus, at concentrations which completely inactivated virus suspensions, had no effect on the activity of the nucleic acid. It is interesting to recall that infectious viral RNA preparations have been shown by several workers to be inactivated by both specific antisera and normal sera. This inactivation is caused, in all probability, by a combination of non-specific protein-RNA interaction and degradation by traces of RNase in the sera. Ito did not report on attempts to isolate and characterize Shope papilloma virus from his DNA-induced papillomas. Whether or not the production of intact virus is a prerequisite for the establishment of an actively growing papilloma in this system is a question which should be answered. It seems at least possible that the DNA might alter a sufficient number of cells to establish tumor growth without the intervention of intact Shope papilloma virus.

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GASEOUS STERILIZATION[1]

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Gaseous sterilization, as it is presently used and understood, is a fortuitous outgrowth of the field of agricultural and industrial fumigation. During the period between World Wars I and II, gaseous compounds were developed to combat insect infestation in grain and grain products. Among the gaseous chemicals so developed were ethylene oxide and methyl bromide. Later, it was found that ethylene oxide reduced the microbial contamination of the treated foods, and this discovery led to the application and issue of patents concerning its formulation and use as a gaseous decontaminant and sterilant. By 1942, the use of gaseous ethylene oxide to reduce the microbial population of spices and gums had been described in several journals of food technology and manufacture. Early in World War II, the U. S. Government deemed it necessary to establish a biological warfare laboratory under the jurisdiction of the Chemical Corps of the U. S. Army. The use of gaseous fumigants as sterilants was reinvestigated by this group, and the results of their investigations with ethylene oxide were published by Phillips & Kaye in 1949 (1 to 4). Since their first paper gave a thorough review of the work on ethylene oxide prior to 1949, no attempt will be made to discuss these early reports. This review will cover much of the work of the past decade, particularly the past four years, and will overlap an extensive review published by Phillips (5) four years ago and a recent brief review by Phillips & Warshowsky (6). Because there is a tendency to become overly concerned with the medical applications of gaseous sterilization, the author will review the applications in the food industry where recent legislation by the federal government has curtailed the use of ethylene oxide on some products.

Introduction

Gaseous sterilization is the treatment of objects or materials with a chemical in the gaseous or vapor state to destroy all microorganisms with which they have been contaminated. The need for such a method of sterilization has developed from the use of many items that cannot be subjected to heat, radiation, or liquid chemical sterilization. The following advantages are noted: sterilization is at low temperatures thus avoiding damage to heat- and moisture-sensitive materials; objects or items can be terminally sterilized in their containers or packages; diffusion of some gaseous sterilants through containers of plastic, paper, or fabric eliminates problems of removal of sterilant; gaseous sterilants penetrate into areas not reached by liquids; sterilization by some gases takes place in the presence of large quantities of

[1] The survey of the literature pertaining to this review was completed in November, 1960.

organic matter; and it is possible to use simple equipment such as plastic or rubber bags and metal drums as sterilizing chambers. The disadvantages are as follows: the length of time for sterilization and aeration is much greater than with other methods; the flammability of some gases requires special operating procedures; toxicity hazards exist with most sterilizing gases; chemical analyses are occasionally necessary to determine residues or addition products formed during sterilization of some organic materials; corrosion or other forms of physical damage can occur from improper sterilizing procedures; and the cost of sterilization is much greater than with moist or dry heat. As a general rule, gaseous sterilization needs close control and supervision to insure effectiveness.

All of the compounds most actively employed in gaseous sterilization are alkylating agents. Brief sketches of some of the properties and the necessary requisites for their use as gaseous sterilants are presented for five alkylating agents in their decreasing order of use: (*a*) Ethylene oxide: C_2H_4O; b.p., 10.4°C.; flammability limits in air, 3.6 to 100 per cent by volume; requires dilution by inert gases, carbon dioxide, or chlorofluorohydrocarbons, for safe use; usual concentrations for sterilizing purposes, 400 to 1000 mg. per l.; requires relative humidity in the range of 25 to 50 per cent; has strong penetrating ability and moderate microbicidal properties; most versatile gas for sterilizing purposes. (*b*) Propylene oxide: C_3H_6O; b.p., 34.0°C.; flammability limits in air, 2.1 to 21.5 per cent by volume; may or may not require dilution with inert gases for safe use; acceptable concentrations for sterilizing purposes, 800 to 2000 mg. per l.; requires humidities in the range of 25 to 50 per cent; penetrating ability and microbicidal properties are less than those of ethylene oxide; frequently used as a substitute for ethylene oxide. (*c*) Formaldehyde: CH_2O; b.p., −21°C. for pure gas, but approximately 90°C. for formalin solutions; flammability limits in air, 7 to 73 per cent by volume, but this hazard is minimized by the large percentage of water usually present; usual concentrations for sterilizing purposes, 3 to 10 mg. per l.; requires high relative humidity, over 75 per cent; has weak penetrating ability but strong microbicidal properties; most commonly used as a surface sterilant. (*d*) Methyl bromide: CH_3Br; b.p., 4.6°C.; essentially non-flammable; usual concentrations for sterilizing purposes, about 3500 mg. per l.; requires moderate relative humidities in the range of 40 to 70 per cent; has strong penetrating ability but weak microbicidal properties; usually employed as a decontaminant for fungi or vegetative bacteria. (*e*) β-Propiolactone: $C_3H_4O_2$; b.p., 163°C.; no flammability hazard at room temperatures; usual concentrations for sterilizing purposes, 2 to 5 mg. per l.; requires high relative humidity, over 75 per cent; has weak penetrating ability but very strong microbicidal properties; essentially a surface sterilant.

The differences among gases as to penetrating ability and humidity requirements for microbicidal activity are striking and must be considered for any intended use of each of these compounds as sterilants. It should be noted that propylene oxide and methyl bromide are more commonly employed as

gaseous decontaminants rather than as sterilants, i.e., they are used to destroy specific groups of organisms such as fungi, yeasts, coliforms, and salmonellae.

Other compounds which have microbicidal activity and which could be used in gaseous sterilization are epichlorohydrin, epibromohydrin, ethylene imine, ethylene sulfide, glycidaldehyde, propylene imine, chloropicrin, and ozone. Difficulty with commercial availability, increased toxicity, or lower microbicidal activity has prevented their practical development and use. Since most gaseous sterilization is carried out with ethylene oxide, this compound will be the focal point of this review.

Biochemistry of Gaseous Alkylating Agents Used in Sterilization

In 1949, Phillips (2) reported data obtained from studies with a group of gaseous alkylating agents which showed that microbicidal activity was directly related to the alkylating activity of each chemical. Thus, ethylene oxide, ethylene sulfide, and ethylene imine are active alkylating agents and have strong bactericidal activity, but cyclopropane, which has the same 3-membered ring structure, is not an alkylating agent and is inert microbiologically. Other compounds, e.g., methyl bromide, which are structurally quite different from ethylene oxide, but which possess similar activity as alkylating agents, were found to be active as vapor-phase bactericides. Later, Phillips (7) reported that bacterial spores, which are hundreds to thousands of times more resistant to heat and chemical disinfectants than are vegetative bacteria, were only slightly more difficult to kill with alkylating agents than were vegetative cells. He suggested that the ability to kill bacterial spores relatively easily was a general property of alkylating agents, which can act through alkylation of sulfhydryl, amino, carboxyl, hydroxyl, and phenolic groups.

Since 1946, there has been tremendous activity in research on the cytotoxic effects of alkylating agents, particularly the nitrogen and sulfur mustards. Because of the close relationship between growth inhibitory activity and tumor induction, representative compounds from other groups of alkylating agents have been tested for their cytotoxic activity. Various epoxides, ethylene imines, methanesulfonates, β-lactones, and diazo compounds have been investigated. These groups of alkylating agents have been referred to as "radiomimetic poisons" because in their biological effects they closely simulate ionizing radiations.

Since there is a wide variety of structures among these groups of alkylating agents, the only common feature is the ability to act as alkylating agents under physiological conditions. A further characteristic of all of these compounds is that they are electrophilic and most readily react with so-called nucleophilic (electron-rich) groups such as organic and inorganic anions, amino groups, and sulfide groups. It is convenient to picture the reactions as usually involving a carbonium ion (8). Esters, alkylated amines, ethers, thio ethers, and ammonium and sulfonium compounds are formed as a result

of these reactions. Under physiological conditions there will be little or no reaction with undissociated acid groups or with ammonium cations since these are not nucleophilic (8).

Stacey *et al.* (9) have reacted bovine serum albumin under physiological conditions with different alkylating agents including *bis*-epoxypropylether and propylene oxide. While all the different types of alkylating agents esterify carboxyl groups, only the epoxides reacted extensively with the primary amino and imidazole groups provided by the side chains of lysine and histidine, respectively. Further experiments (9) with two model substances that have only one kind of reactive group, the polyacid, polymethacrylic acid, and the polybase, polyvinyl amine, confirmed the fact that, while all the reagents used esterify carboxyl groups, only the epoxides reacted extensively with the amino groups.

Contrary to these results, Fraenkel-Conrat (10) earlier had claimed that more than 50 per cent of the carboxyl groups of egg albumin and β-lactoglobulin were esterified by propylene oxide. His method of estimation consisted of a determination of the amount of basic dye which combines with the protein at pH 11.5 after standing for a least 24 hr. There is ample evidence to show that the ester groups in proteins are completely saponified under these conditions, and therefore this method of analysis cannot be used for determining esterification of carboxyl groups (11).

Windmueller *et al.* (12, 13) investigated the reaction of ethylene oxide with nicotinamide and nicotinic acid, and with histidine, methionine, and cysteine. These studies were directly related to the possible fate of these compounds when foods are fumigated with ethylene oxide. Ethylene oxide was found to react with nicotinamide in aqueous solution at 25°C. to yield, after acidification with HCl, N-(2-hydroxyethyl)-nicotinamide chloride. By a similar reaction, nicotinic acid is converted to the betaine of N-(2-hydroxyethyl)-nicotinic acid. Ethylene oxide reacted with imidazole or histidine to yield the corresponding 1,3-*bis*-(2-hydroxyethyl)-imidazolium derivative. With methionine or N-acetylmethionine, ethylene oxide hydroxyethylates the sulfur to yield the corresponding sulfonium derivatives. Likewise, double alkylation of the mercapto group of cysteine produces a sulfonium group. The primary amino groups of these amino acids also become alkylated, but esterification of carboxyl groups does not seem to be involved. Thus, all of the reactions involved hydroxyethylation of an atom with one or more lone pairs of electrons, either nitrogen or sulfur.

As a result of their work, Windmueller *et al.* (12) came forth with a hypothesis to explain the moisture requirements for ethylene oxide sterilization. Their proposal is that water facilitates proton reactions, and protons are required by an alkylating agent sucn as ethylene oxide in its reaction with tertiary nitrogen compounds. It is my opinion that whether an alkylating agent has a low or high relative humidity requirement for activity as a gaseous sterilant may also be related to whether the agent acts by first-order or second-order nucleophilic substitution reactions. Since this is a very complex subject, the reader is referred to the article by Price (14).

There is the possibility that alkylating agents produce their characteristic cytotoxic effects by enzyme inhibition. Phosphokinases, certain peptidases, choline oxidase, and acetylcholine esterase have proved to be among the most sensitive enzymes so far examined. The objection to the enzyme inhibition theory has been that the concentrations of alkylating agent required for inactivation do not appear to be achieved *in vivo* when cytotoxic effects can be demonstrated (15). All alkylating agents appear to react readily with sulfhydryl groups of proteins and enzymes although in the native protein not all sulfhydryl groups are accessible (9).

Alexander & Stacey (16) hold the view that the biologically important reaction of alkylating agents occurs with DNA (or possibly RNA) in nucleoproteins and modifies these in such a way that they become useless to the cell. The only way by which preformed DNA can be modified is by reaction with the phosphate groups, which constitute the Achilles heel of this substance. The predominant reaction of epoxides with DNA is one of esterification of the phosphate groups and follows second-order reaction kinetics (9). After long treatments with excess epoxide (propylene oxide and hexane bisepoxide), changes in the ultraviolet absorption spectrum are noticed. There is increased absorption at 270 mμ, which suggests reaction with groups other than phosphate, such as the amino group of guanidine or one of the ring nitrogens of the purines or pyrimidines (9). As a result of their investigations with both alkylating agents and radiation, Alexander & Stacey (16) feel that the biological damage of ionizing radiations and alkylating agents is initiated at the molecular level by an attack on the DNA moiety, although by different chemical reactions. It may be that the radiomimetic properties of alkylating agents account for the high microbicidal activity of these compounds and their consequent use as gaseous sterilants.

Biological Effects of Gaseous Alkylating Agents

During the past 15 years there has accumulated a vast amount of data which show that alkylating agents are mutagenic, that they damage the cytoplasm and nuclei of rapidly growing cells, and that they cause injury to the chromosomal mechanism of rapidly dividing cells. These effects are usually noted in cells that are exposed to near-lethal concentrations of these agents. The desired goal in gaseous sterilization is the lethal effect on microorganisms, and there is little interest in the biological events that occur during this disinfection process. Since mammalian cells are as liable as microorganisms to destruction by these chemicals, their toxic properties are of direct concern to man. Accordingly, a brief discussion of their mutagenicity, carcinogenicity, toxicity, and microbicidal properites is relevant.

The mutagenic action of alkylating agents has been recorded for bacteria, yeasts, molds, barley seeds, and root tips of leguminous seedlings. Strauss & Okubo (17) state that protein synthesis is not required for the fixation of potential mutations in *Escherichia coli* by the alkylating agents, ethyl sulfate and epichlorohydrin. Kölmark & Giles (18) tested six different monoepoxides as inducers of reverse mutations in a purple adenineless strain of *Neurospora*

crassa. In general, they found that epoxy rings carrying side chains with strong electronegative properties are stronger mutagens than epoxy compounds with weak electronegative side chains. Ehrenberg & Gustafsson (19) have treated barley seeds with ethylene oxide and found it to be a mutagenic agent of equal or even greater efficiency than ionizing radiation; ethylene imine (20) is even more efficient as a mutagenic agent than ethylene oxide. β-Propiolactone causes reversions to growth factor independence and gene mutation in *N. crassa* (21) and produces chromatid aberrations in lateral root-tip chromosomes of *Vicia faba* (22). Iyer & Szybalski (23) have found β-propiolactone to be a very efficient mutagen for reversion to growth factor independence in *E. coli.*

After the discovery of the radiomimetic properties of alkylating agents, several of these compounds were tested for and were found to possess carcinogenic activity (24). These findings were not noticed by the technical people doing research in gaseous sterilization until the proposed use of β-propiolactone as a gaseous sterilant for enclosed spaces was questioned by federal regulatory agencies and their scientific advisors (25, 26). β-Propiolactone has been found to be an initiator of skin sarcomas and carcinomas in the mouse (27, 28, 29), and Wisely & Falk (30) have stated that single intradermal doses of 0.002 ml. in mice have caused sarcomas and squamous papillomas. The mention of the production of sarcomata in rats with diepoxybutane by Koller (31) and the confirmation if its carcinogenic activity by McCammon *et al.* (32) caused speculation that the monoepoxides, ethylene oxide and propylene oxide, would also be found carcinogenic. Walpole (24) has recorded the induction of sarcomata in rats after repeated injection of propylene oxide but not after treatment with ethylene oxide. He has concluded from his investigations that the presence in a molecule of one or more groups having alkylating function may result in carcinogenic activity. It certainly behooves the users and practitioners of gaseous sterilization not only to be concerned with the acute and chronic toxicity of gaseous alkylating agents but also to keep all contacts with these compounds to a minimum to prevent any concern about the possibility of long-term biological effects.

The acute toxicity of ethylene oxide and its irritant action on the skin and eyes are well known. Pure anhydrous liquid ethylene oxide does not produce primary injury to the dry skin of workers, but solutions of ethylene oxide have a vesicant action (33, 34) and may cause conjunctivitis if splashed in the eye (35, 36). Burns are particularly likely to occur when the solution is held in contact with the skin by clothing, gloves, and shoes. In common with many other skin irritants, ethylene oxide can produce sensitization (34).

Skin lesions can result from contact with ethylene oxide absorbed in sterilized rubber gloves and shoes (1, 37). Royce & Moore (37) have shown that immediately after overnight treatment in liquid ethylene oxide a piece of rubber weighing 1 gm. contained no less than 600 mg. of ethylene oxide. After sterilization in 10 per cent ethylene oxide gas, 1 gm. of rubber contained 15.4 mg. of the oxide; at 4 hr. there was 0.4 mg. remaining. A shoe can absorb up to 10 gm. of ethylene oxide during sterilization with this compound (38), and Beard & Dunmire (39) have used radioactive ethylene oxide

to determine which parts of shoes are most retentive. Grundy *et al.* (40) have established that ethylene oxide gas absorbed during the sterilization of plastic intravenous injection equipment exerted a continuing sterilizing effect after the materials were removed from the sterilizer. These data point to the necessity of holding treated plastic and rubber materials at least 8 hr. or preferably a day before use.

Several recent papers have presented the results of animal experiments dealing with the acute and chronic effects of both ethylene oxide and propylene oxide vapors. When animals were subjected to repeated 7-hr. exposures to ethylene oxide vapor, 5 days a week, for 6 or 7 months, guinea pigs, rabbits, and monkeys tolerated 113 p.p.m., and rats aad mice tolerated 49 p.p.m. without adverse effects (41). A concurrent independent study (42) observed that dogs, rats, and mice exposed over a 6-month period to 100 p.p.m. of ethylene oxide exhibited no clinical signs of toxicity, but a few significant hematological changes were noted in one animal. These same investigators (42, 43) concluded from toxicity studies with propylene oxide that it was about one-third as toxic as ethylene oxide by inhalation and ingestion in all animal species studied. They recommended an industrial hygiene standard of 50 p.p.m. for ethylene oxide vapor and 150 p.p.m. for propylene oxide vapor. Those readers interested in the toxicity of other gaseous alkylating agents should consult the article by Rowe (44) on methyl bromide, the paper by Feazel & Lang (45) on β-propiolactone, and any good toxicology handbook for the toxicity of formaldehyde vapor.

Most of the available reports concerned with the microbicidal properties of gaseous alkylating agents present data that relate the physical factors of time, temperature, relative humidity, and concentration of gas to the destruction of a given population of microorganisms. Only the report of Loveless & Stock (46) on the inactivation of T_2 phage by aqueous alkylating agents describes in part how mono- and *bis*-epoxides react with a microorganism. Their data support many of the biochemical mechanisms of alkylating action previously discussed and may promote more basic research to learn how ethylene oxide inactivates microorganisms.

Ethylene oxide is effective against all microorganisms. The earlier literature containing references to its effect on many bacteria and molds as natural contaminants has been reviewed by Phillips & Kaye (1). Phillips (2, 7) was the first to quantitate the relative resistance of spore-forming and vegetative bacteria to ethylene oxide. He found that spores of *Bacillus globigii* (now *B. subtilis* var. *niger*) were only 2 to 6 times more resistant than vegetative cells from several bacterial species. Although no quantitative data are available for the relative resistance of *Mycobacterium tuberculosis* to ethylene oxide, several papers (47 to 50) have verified its destruction on contaminated materials. Friedl *et al.* (51) tested the sporicidal activity of ethylene oxide against five aerobic and five anaerobic spore-forming bacterial species employing the sporicide testing method of the Association of Official Agricultural Chemists. Dried spores, which were much more resistant to ethylene oxide than were wet spores, from all test organisms were destroyed after an 18-hr. exposure. Under their conditions, spores of *B. subtilis* and *Clostridium*

sporogenes withstood a 6-hr. exposure, while spores of *B. globigii* and *B. anthracis* were killed in 30 min. No direct relation could be found between resistance to constant boiling HCl and to ethylene oxide. Although there have been occasional reports of the destruction of staphylococci only after prolonged exposure to ethylene oxide (52, 53), the data available from studies that were designed to compare the resistance of bacterial spores and staphylococci have shown bacterial spores to be more resistant (2, 50, 54, 55). Toth (56) has recorded aerobic sporeformers to be more resistant than vegetative bacteria, yeasts, and molds to destruction by ethylene oxide. Church *et al.* (57) have pointed out that populations of spores of some aerobic bacilli are heterogenous in their susceptibility to ethylene oxide and consist of a major component sensitive to ethylene oxide and a minor component resistant to ethylene oxide.

The sterilizing activity of ethylene oxide for viruses has been confirmed by several investigators. The following viruses have been inactivated by liquid ethylene oxide: vaccinia (47), influenza A and B (58), Newcastle disease virus (58), Theiler's mouse encephalomyelitis virus (58), MM mouse encephalomyelitis virus (58, 59), lymphocytic choriomeningitis virus (59), Eastern equine encephalomyelitis virus (59, 60), and foot-and-mouth disease virus (61, 62). The activity of gaseous ethylene oxide against 15 animal viruses was noted by Mathews & Hofstad (63), against foot-and-mouth disease virus by Savan (64), Callis *et al.* (65), and Fellowes (62), and against Columbia SK encephalomyelitis virus and vaccinia virus by Klarenbeek & van Tongeren (66).

The microbicidal properties of the other alkylating agents employed in gaseous sterilization have not been quantitated to the same extent as have those of ethylene oxide. Phillips (5) has adequately noted the bactericidal properties of gaseous formaldehyde. The sporicidal activity of methyl bromide against spores of *B. anthracis* has been documented by Kolb & Schneiter (67) and Kolb *et al.* (68) and against fungi by Munnecke *et al.* (69). Phillips (5) states that methyl bromide has one-tenth the activity of ethylene oxide or approximately the activity of 10 per cent ethylene oxide inerted with 90 per cent carbon dioxide by weight. Bruch & Koesterer (70) have quantitated the sporicidal activity of propylene oxide, which has about one-fourth the activity of ethylene oxide. The microbicidal properties of β-propiolactone are recorded in several papers from the U. S. Army Biological Warfare Laboratories (71, 72, 73). On a Ct_{90} basis (molar concentration of gas times the time for 90 per cent kill), β-propiolactone is approximately 25 times more active as a vapor-phase disinfectant than formaldehyde, 4000 times more active than ethylene oxide, and 50,000 times more active than methyl bromide (71).

To date, all evidence indicates that the effect of ethylene oxide or the other alkylating agents employed in gaseous sterilization is irreversible and microbicidal. However, Morpurgo & Sermonti (74) have demonstrated the reactivation by manganous chloride of spores of *Penicillium chrysogenum* inactivated by nitrogen mustard. They suggest that the site of the inactiva-

tion-reactivation process is in the cytoplasm of these spores. If their work is confirmed, this effect should be sought following the inactivation of organisms by other alkylating agents.

Physical Handling and Mode of Use of Gaseous Sterilants

Sterilization with gaseous ethylene oxide is a function principally of the concentration of gas, time of exposure, and temperature of exposure. Humidity must be known and controlled within certain limits, i.e., a certain minimal amount of moisture must be present for sterilization to occur. The degree of contamination constitutes another important variable since organisms protected by layers of dense organic material such as dried pus, blood, and serum or entrapped within crystals of salt or the dried solids from thick microbial suspensions can resist destruction by this method. The molecules of gas plus some molecules of added water or of water already present in the material must be in contact with an organism for inactivation to occur.

As a result of the report by Kaye & Phillips (4), it is generally accepted that ethylene oxide sterilization proceeds better at relative humidities of from 25 to 50 per cent than at humidities approaching saturation. These data support the empirical use of ethylene oxide in the presence of no added moisture by food processors (52, 75, 76, 77). These processors rely on the available water present in the dried powders or flakes to provide the necessary water for the inactivation reaction. The difficulties experienced in the destruction of excessively desiccated organisms (4, 63) can be traced to the lack of water in the cells or to the inability of added water to penetrate the dry cells. Opfell *et al.* (78) have shown that the absence of hygroscopic substances in the drying menstruum of spores of *B. globigii* increased the resistance of these spores to sterilization by ethylene oxide. Znamirowski *et al.* (55) have claimed that lack of humidity control was responsible for the inefficiency of a commercially available ethylene oxide sterilizer, but an analysis of their methods and data indicates that entrapment of spores in salt crystals provides a more likely explanation for the failure to achieve sterility.

Ethylene oxide concentrations are usually expressed as milligrams per liter (mg. per l.) or ounces per 1000 cubic feet of chamber space. In a series of experiments with ethylene oxide at concentrations of 22 to 884 mg. per l. and temperatures of 5 to 37°C., Phillips (2) found that the death rate was logarithmic, that doubling the concentration of ethylene oxide roughly halved the time required for sterilization, and that the temperature coefficient of the reaction was 2.7 for each rise of 10°C. Although the exact time for sterilization depends on the volume of the load, the nature of the materials and their degree of contamination, our laboratory employs 720 mg. per l. for 8 hr. at 100°F. (37.7°C.) or for 4 hr. at 130°F. (54.4°C.) for a load of clean plastic items in a 24-cubic foot sterilizer. These times are increased several fold for loads of greater volume in larger sterilizers.

Any gaseous sterilization process should be controlled by the use of biological indicators placed in the most difficult article or location in an article to be sterilized. Spores from any of several spore-forming bacterial species

can be employed, but unpublished data from our laboratory have shown that spores from certain strains of *B. subtilis* and *C. sporogenes* are highly resistant. Friedl *et al.* (51) have also described these two organisms as being highly resistant to destruction by ethylene oxide. The use of spores with high heat resistance is not necessary since no correlation has been found between resistance to heat and gaseous sterilization. The concentration of organisms used should be reasonable (not greater than 1×10^6 spores dried on a 2-in. by $\frac{1}{4}$-in. filter paper strip) because physical protection from dense concentration can result. Royce & Bowler (79) have recently reported the use of a chemical control device that has been correlated against biological inactivation by ethylene oxide. This device requires sorption and penetration of its plastic package by ethylene oxide before the gas can react with the chemical reagents.

Although the penetration and diffusion of ethylene oxide through materials such as flour, spices, and layers of paper, clothing and blankets has been documented in past reviews (1, 5, 6), much more data are needed on the sorption by and penetration through many plastic films and articles. Among the inexperienced, it is not uncommon to attempt to sterilize articles wrapped in plastic films that are nearly gas- and moisture-proof. Waack *et al.* (80) have determined the permeability constants for several polymer films to ethylene oxide, methyl bromide, and other gases. Results of their studies showed that polyethylene and rubber hydrochloride (see original article for trade names) were most permeable to ethylene oxide, whereas cellulose acetate, polyvinylidene chloride, and polyester were less permeable to ethylene oxide in the order listed. Dick & Feazel (81) in the development of flexible plastic chambers for ethylene oxide sterilization tested the relative permeability of several plastic films and found cellophane, polyvinyl alcohol, and polyester impermeable to ethylene oxide under their test conditions. The results of Lloyd & Thompson (82) with wrapping materials such as cellophane, polyvinylidene chloride, polyethylene and laminated polyester indicated that polyvinylidene chloride was the most difficult and polyester rather difficult to penetrate with ethylene oxide. Unpublished data from our laboratory indicate that polyethylene, which is highly permeable to ethylene oxide, has a low transmission rate for water vapor, but since the humidity requirement for ethylene oxide sterilization is low, enough water vapor is usually present in the packages to allow satisfactory sterilization.

Although liquid ethylene oxide is stable to detonating agents (36), the vapor is explosive and will propagate a flame at concentrations ranging from 3.6 per cent by volume in air to 100 per cent ethylene oxide (36, 83). This flammability problem can be avoided by dilution with inert gases such as carbon dioxide (84) or chlorofluorohydrocarbons (83, 85). Definite nonflammable compositions have been formulated, and many of these gaseous mixtures are commercially available. This safety is achieved at a considerable loss in potency since about 90 per cent of these mixtures is diluent. This loss of potency is less for the chlorofluorohydrocarbon mixtures (11 per cent by weight ethylene oxide and equal parts by weight of dichlorodifluoromethane

and trichloromonofluoromethane) since the concentration of ethylene oxide attained with these mixtures at 1 atm. pressure is almost three times that obtained with carbon dioxide mixtures (10 per cent ethylene oxide by weight).

A wide variety of equipment has been used for ethylene oxide treatments. Any gas-tight container can be employed although rigid chambers similar to laboratory autoclaves are usually used. The use of large vacuum chambers from which air is first removed before pure ethylene oxide is admitted has been described for the decontamination of bulk food materials (52, 75, 76, 77, 86, 87). The conversion of ordinary laboratory autoclaves (49, 88, 89) and the use of plastic bags (81, 89), tarpaulins (38, 89), and metal drums (38, 89) have been reported. Specially designed, automatic equipment is now commercially available (90, 91, 92). Recently, in West Germany, commercial equipment has become available that employs the 10 per cent ethylene oxide-90 per cent carbon dioxide mixture at pressures of 3 to 6 atm. and temperatures of 35 to 65°C. (50, 93). This procedure has been called the "Mainz sterilization method" and appears to have few advantages over the processes that employ the chlorofluorohydrocarbon mixtures at much lower pressures and at similar ethylene oxide concentrations.

The physical handling and mode of use of other gaseous sterilants can be given only brief mention. The sterilization of rooms or large spaces with formaldehyde has been reviewed before (5), and its recent use in disinfecting chambers has been detailed by several European workers (94, 95, 96). The employment of β-propiolactone as a surface sterilant in small chambers, rooms, or buildings has been cited in several recent publications (25, 26, 71, 97, 98). Propylene oxide (70, 99) and methyl bromide (67, 68, 69) are utilized in the same type of equipment as ethylene oxide.

Medical Applications of Gaseous Sterilization

It has been documented in past reviews that ethylene oxide will sterilize hospital bedding, plaster of Paris bandages, various articles of clothing and footwear, plastic and rubber laboratory items, ophthalmic instruments, catheters, cystoscopes, bronchoscopes, forceps, extractors, scalpel blades and holders, clinical thermometers, dental and root canal files, and penicillin in powder or solution. These accepted uses and the newer applications for ethylene oxide sterilization in hospital practice have also been discussed by Spaulding *et al.* (100), Freeman & Barwell (54), and Thomas (101).

Recent applications are included in the reports of Skeehan (102) and Linn (103), who have sterilized ophthalmic instruments in a small, table-model, ethylene oxide sterilizer. This same model of sterilizer has been criticized as being inefficient (104), and another group (100) has doubled the manufacturer's recommended exposure time. Other new or confimed applications are the sterilization of blankets (53), urological instruments (105), plastic intravenous injection equipment in commercial packages (40), ampules for anesthesia (106), procaine hydrochloride tablets (107), and the decontamination of blankets by the addition of ethylene oxide to plastic bag containers (108). Additional uses that are known to the reviewer include the

sterilization of surgical rubber gloves, sutures, plastic syringes, disposable needles, oxygen tents, inhalation equipment, cameras, and photographic film.

A new application that has received much attention is the sterilization of pump-oxygenators. Spencer & Bahnson (109) modified a standard steam autoclave to sterilize a completely assembled pump-oxygenator with the ethylene oxide-chlorofluorohydrocarbon mixture. McCaughan *et al.* (110) made a 61-cubic ft. stainless steel chamber which employs the ethylene oxide-chlorofluorohydrocarbon mixture at a concentration of 180 to 200 mg. per l. Completely reliable sterilization of an assembled vertical screen pump-oxygenator was obtained at 16-hr. exposure at room temperature and ambient humidities. Bracken *et al.* (111) have used large polyvinyl chloride bags and the 10 per cent ethylene oxide-90 per cent carbon dioxide mixture to sterilize a heart-lung machine. They eventually hope to simplify the sterilization technique by discarding the plastic bag altogether and to maintain an adequate concentration of ethylene oxide in the machine by a slow trickle, i.e., the oxygenator would become its own sterilizing chamber.

To the reviewer's knowledge gaseous formaldehyde is very seldom used in the United States to sterilize hospital equipment and supplies or to fumigate hospital rooms. However, in Great Britain the committee on formaldehyde disinfection of the Public Health Laboratory Service has recently submitted a lengthy report on disinfection of hospital items by gaseous formaldehyde (95). This committee concluded that formaldehyde gas could not be recommended for disinfection of fabrics contaminated with smallpox virus or anthrax spores, nor was it really suitable for the disinfection of articles contaminated with tubercle bacilli. Contrary to these findings Caplan (96) has reduced the incidence of infections in a surgical ward by the regular disinfection of blankets and bedside curtains with formaldehyde vapor. Thomas *et al.* (53) compared sterilization of blankets by several methods. Although they list boiling as the simplest way of "sterilizing" blankets, ethylene oxide was considered a more promising sterilizing agent than formaldehyde, which, in normal use, allowed the survival of non-sporulating organisms. Phillips (5) in past reviews has noted the deficiencies of formaldehyde disinfection (condensation of gas and lack of penetration) and has pointed out how superior ethylene oxide is for many of these hospital applications.

Another gaseous alkylating agent that is felt to have potential applications in the hospital field is β-propiolactone. Like formaldehyde, this gas is non-penetrating and essentially a surface sterilant. After Hoffman & Warshowsky (71) detailed its microbicidal properties and mentioned its use to decontaminate a barracks, several reports on its use in hospital rooms followed (25, 26, 27). In general, these reports have shown that rooms can be treated and put back into use within 24 to 48 hr. The commercial development of this application has been hindered by the discovery of the carcinogenic properties of this compound on mouse skin as previously discussed. An additional limited application is the sterilization by the lactone of surfaces of unwrapped ophthalmic instruments in plastic sacks, desiccators, and plastic or metal boxes (98).

AGRICULTURAL AND INDUSTRIAL APPLICATIONS OF GASEOUS STERILIZATION

Although gaseous sterilization with ethylene oxide had its birth in the food industry, much of the information regarding this application has remained a trade secret or has been listed briefly in patents. Very little information has been published in the United States during the past decade that describes new applications for ethylene oxide sterilization in this field. Some data on existing and known uses were presented by Hall (86), Pappas & Hall (87), and Coretti (112) verifying the destruction of thermophiles, yeasts, and molds in food ingredients. What is new and what has brought a sharp turn of events in this field has been the examination of the treated foods toxicologically and the promulgation of regulations by governmental agencies regarding acceptable levels of addition products and residues.

The first reference to nutritional damage of foods treated with ethylene oxide was presented in the paper by Hawk & Mickelsen (113), who noted severe growth depression in weanling albino rats when their diet, either stock or purified, had been exposed to the gas. Thiamine was indicated to be one of the factors, though not the only one, destroyed by this treatment. Oser & Hall (114) followed with evidence that when dried yeast and a rat diet composed of natural products were exposed to ethylene oxide according to a commercial method, there was no significant destruction of thiamine, riboflavin, nicotinic acid, pantothenic acid, or choline. There was an indication that some folic acid and pyridoxine were destroyed. The reviewer cannot agree with their objections that the levels of treatment used by Hawk & Mickelsen were too severe since gas concentrations in food sterilization can range from 300 to 1000 mg. per l. (75, 76, 115) and Griffith & Hall report the use of 1600 mg. per l. in one of their patents (116).

Further work by Bakerman *et al.* (117) has shown that various vitamins were destroyed when mixtures of the B vitamins suspended in starch were exposed to ethylene oxide. Practically all of the thiamine and large amounts of riboflavin, pyridoxine, niacin, and folic acid were destroyed when choline chloride was present; there was no destruction of pantothenic acid, biotin, or vitamin B_{12}. The effect of ethylene oxide on thiamine was due in part to the increase in pH in the presence of choline chloride; the destructive effect of ethylene oxide on thiamine solutions at pH 9 has also been shown by Diding (107). This alkalinity may explain the destruction of thiamine but cannot explain the destruction of niacin, riboflavin, or folic acid.

Windmueller *et al.* (118) observed that exposure of casein to very high concentrations of ethylene oxide resulted in essentially complete destruction of histidine and methionine determined by rat feeding studies and microbiological assays. As a result of their work, Mickelsen (119) ran microbiological assays on purified diets exposed to ethylene oxide under conditions that he previously described (113) and found that 22 per cent of the histidine and 17 per cent of the methionine were destroyed.

The realization that a tertiary nitrogen group was common to six of the

labile nutrients prompted Windmueller *et al.* (12) to investigate the reaction of ethylene oxide with pyridine and the closely related nicotinamide and nicotinic acid. The destructive action of ethylene oxide on histidine, methionine, cysteine, and lysine was also studied in model systems in which the individual amino acids or amino acid derivatives were treated with ethylene oxide in aqueous solution (13). Nutritional damage by ethylene oxide treatment appears to be correlated with the electrophilic hydroxyethylation of an atom with one or more lone pairs of electrons, either nitrogen or sulfur.

With the passage in 1958 of the Food Additives Amendment to the U. S. Food, Drug and Cosmetic Act, the use of direct and indirect food additives not approved by the U. S. Food and Drug Administration was to be halted after March 6, 1960, although temporary extensions until March 6, 1961, could be granted. Ethylene oxide is considered a direct additive to foods treated with it and has been granted temporary clearance until March 6, 1961, for use in dried fruits, ground spices, dried mushrooms, and edible gums (120, 121). The objection to the use of ethylene oxide stems not only from the destruction of vitamins and amino acids but also from the known toxicity of ethylene and diethylene glycols that are almost invariably formed in small quantities as the hydrolytic residues from ethylene oxide sterilization of foods. Thus, the food industry has started to search for new sterilants, is reexamining other compounds such as propylene oxide and methyl bromide, and is developing new chemical procedures for residue determinations.

Gordon *et al.* (122) fumigated a prune with C_{14}-ethylene oxide and determined the main sites of alkylation. They found over 50 per cent of the total radioactivity to be combined with insoluble hydroxyethyl cellulose in the prune skin, about 30 per cent as hydroxyethylated sugars in the pulp, 10 per cent as alkylated proteins and amino acids, and 3 per cent as glycols. A similar study has been carried out by Winteringham and associates (123, 124, 125) with C_{14}-methyl bromide and wheat flour. The data from these papers indicate that no toxic products per se are formed, but only animal feeding studies could confirm this supposition.

Other new uses for gaseous sterilants in the agricultural field include the sterilization of dried gelatin and dried eggs by ethylene oxide or its mixture with propylene oxide or methyl bromide (126). Although Adam (127) has confirmed the destruction of salmonellae in egg powder by this method, he urges caution in the adoption of this technique because of possible toxicological and nutritional effects. The decontamination of various seeds with ethylene oxide has been described by Steinkraus *et al.* (128), and the destruction of fungi in barley seeds by propylene oxide has been recorded by Tyner (129). McBean & Johnson (130) and Nury *et al.* (131) have reported the preservation of prunes with propylene oxide, and Bruch & Koesterer (70) have utilized propylene oxide to decontaminate flaked and powdered foods.

While sterilization of powders is a problem in the pharmaceutical industry, many of the details of their techniques are kept as trade secrets. Bullock & Rawlins (132) have detailed the sterilization of kaolin powder mixtures by gaseous formaldehyde moving through the powders at constant flow rates or

constant gas concentrations. They showed that the death rate of spores of *B. subtilis* is a function of the amount of formaldehyde permeating the powders in unit time and that humidities over 85 per cent are required for this process. A similar apparatus was used by Abbott *et al.* (133) to expose crystals formed from liquids contaminated with spores of *B. subtilis* to sterilization by gaseous formaldehyde and ethylene oxide. Neither of these gases could sterilize crystals which had spores entrapped in the crystal matrix. The static sterilization of talc powder in powder cans by the injection of propylene oxide or ethylene oxide has been listed in a recent patent by Masci (134).

Other recently noted applications for formaldehyde include the decontamination of microbiological laboratories (135) and incubators in egg hatcheries (136). These uses for formaldehyde will eventually be assumed by β-propiolactone vapor. Several investigators have demonstrated the decontamination of large industrial laboratories with this compound (25, 26). The full usefulness of β-propiolactone will not be achieved until its toxicity is further defined.

Future

After an attempt at some representation of the total picture of the field of gaseous sterilization, a projection into the future reveals many bright areas contrasted with some uncertain areas which need delineation and clarification. On the promising side is the fact that the use of ethylene oxide in the hospital and medical field will grow. The increasing use of disposable plastic materials requires their sterilization by a "cold" method. As specialized apparatus such as heart-lung machines become more prevalent in hospitals, the installation of permanent gaseous sterilization equipment will follow. The problems of staphylococcal contamination in many hospitals has forced numerous "infections committees" to consider sterilization of blankets, linens, and mattresses with ethylene oxide and even decontamination of patients' rooms with β-propiolactone or formaldehyde. Thus, the potential applications for gaseous sterilization look very bright and hopeful in this area.

In contrast, the future of gaseous sterilization in the food and agricultural field is certainly cloudy and befuddled. Although the use of gaseous chemicals as fumigants (destruction of insects and fungi) is a permanent part of the agricultural scene, the application of these same gaseous chemicals for sterilization of food or materials that may come into contact with foods remains to be cleared with governmental regulatory agencies. It may well be that the use of the available gaseous sterilants will have to be closely controlled with respect to the type of foods treated and the degree of decontamination that would be acceptable. Certainly, the presence of pathogenic staphylococci and salmonellae in many dried foods necessitates some remedial action. Part of the uncertainty regarding gaseous sterilization of food results from lack of knowledge of possible toxic or damaging effects, but, as more information is accumulated about the toxicological, cytotoxic and tumor-inhibiting effects of alkylating agents, the present difficulties will certainly be resolved.

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ANTIGENIC VARIATION IN UNICELLULAR ORGANISMS[1,2]

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"These are much deeper waters than I had thought"[3]

In preparing this review we have seen no merit in trying—even if it were possible—to survey the vast mass of published data on antigenic variation in unicellular organisms. Our aim has been to establish the underlying causes of such variation, and we will confine the discussion to examples of antigenic variation in the Eubacteria or Ciliata in which something is known of the genetic basis of the variations. These cases are surprisingly few, and in even fewer of them is there any information available on the chemical basis of the variation. We will further confine the discussion to the surface antigens, that is, those cell antigens which react directly with antibodies without any prior disintegration of the cell. These surface antigens are either in the form of appendages proceeding radially from the cell surface (flagella and fimbriae in bacteria or cilia in protozoa), or in the form of layers arranged concentrically around the cytoplasmic membrane (cell wall, microcapsule, and capsule in bacteria—these have been reviewed recently by Salton (105)—and the slime layer in protozoa). The outermost layers are the most important antigenically and they are often impermeable to molecules of the size of antibodies. Inner layers, although antigenic, will be occluded in the intact cell. Thus, the bacterial cell wall is impermeable to antibodies and prevents their combination with the cytoplasmic membrane (137).

Granted these restrictions, we have attempted to consider the types of mechanism by which antigenic variation might occur in the unicellular organisms. This has necessitated a consideration of the chemical basis of antigenic specificity, together with the possible pathways of antigen synthesis and the ways in which these pathways might be affected by changes in genotype. There are many different disciplines involved in such a study and we have been both stimulated and appalled by the way our different scientific (or unscientific) upbringings have caused us to interpret data in a divergent manner. Some of our arguments have been resolved during the course of writing this review while others have remained in a state of uneasy "cold war." We hope that the reader will be made to realise, if he does not realise it already, how much remains to be done and how little has been accomplished.

[1] The survey of the literature pertaining to this review was concluded in October, 1960.

[2] The following abbreviations will be used: DNA (deoxyribonucleic acid); TSP (type-specific polysaccharide); UDPG (uridine diphosphate glucose); UDPGA (uridine diphosphate glucuronic acid); UDPGalA (uridine diphosphate galacturonic acid).

[3] *The Memoirs of Sherlock Holmes*, A. Conan Doyle.

BIOCHEMICAL ASPECTS OF ANTIGENIC VARIATION

Initially, it may be helpful to summarise the types of compound which act as antigens in unicellular organisms. Chemically, they may be divided as follows:

Antigens of single molecular species.—These are usually polypeptide or polysaccharide. They can be divided into homopolymers consisting of a single monomer subunit and heteropolymers consisting of more than one monomer subunit.

Homopolypeptides.—These sometimes occur as antigenic capsules, e.g., the polyglutamic acid capsules of *Bacillus* species in which the monomer unit may be either D- or L-glutamic acid. Antibodies may be specific for the L polymer, the D polymer or the copolymer of L and D (136).

Homopolysaccharides (homoglycans).—Examples are dextrans, the Vi antigen [N-acetyl galactosaminuronic acid units (45)] and colominic acid (N-acetylneuraminic acid units) (6, 7).

Heteropolypeptides.—These commonly form capsules or microcapsules, particularly in Gram-positive bacteria. Usually they are built up from a rather restricted range of amino acids. Examples are the M antigens of streptococci and flagellin, the molecule accounting for at least 99 per cent of the flagellar mass in *Proteus vulgaris* (65).

Heteropolysaccharides (heteroglycans).—These are common as capsules where they usually contain two to four different sugar components. Well-known examples are the capsular antigens of smooth pneumococci and the K antigens of mucoid enterobacteria.

Complex antigens.—These occur as high molecular weight complexes of more than one molecular species. The most important antigenically are the lipopolysaccharide-protein complexes occurring in the cell wall or microcapsule which are typified in the O antigens of the enterobacteria [reviewed by Westphal (140, 141)]. In these complexes the polysaccharide component acts as the antigenic determinant. The basic cell wall mucopeptide of bacteria and the mucopolysaccharides of mammals as typified by the blood group substances are other examples.

The Chemical Basis of Antigenic Specificity

Antigenic variation involves the loss, gain, or change in a particular antigenic group, usually by a loss, gain, or change in one of the polypeptide or polysaccharide antigens discussed previously. Before dealing with the ways in which this may occur, it is important to summarise what we mean by determinant antigenic groupings and to outline the types of structure which are responsible for antigenic specificity. The major part of our knowledge about the chemical basis of the specificity of naturally occurring antigens has been derived from studies on polysaccharides. This has come about because of their importance as antigens as well as their relative structural simplicity compared to proteins. Many studies, particularly those of Goebel,

Heidelberger, Morgan, and Kabat on glycogens, dextrans, and pneumococcal and blood group polysaccharides have confirmed two important aspects of the immunochemical specificity of polysaccharides, i.e. (*a*) the terminal sugars on a polysaccharide chain or side chain are of prime importance in determining specificity and (*b*) the sugars next to the terminal one are of decreasing importance for specificity, although antibodies against a particular polysaccharide are probably heterogeneous in this respect. The maximum size of the antibody-combining site probably varies with different polysaccharides but in the case of dextran it has been found by Kabat (62) to be a terminal chain of six glucose units.

To amplify these points for the more complex heteropolysaccharides characteristic of most microbial species, the recent elegant work by Lüderitz, Kauffmann, Staub, Westphal and their co-workers on the O antigenic polysaccharides of the *Salmonella* should be cited [reviewed in (120, 141)]. In the salmonellas, the properties of the somatic O antigen allow a subdivision into various groups according to the Kauffmann-White scheme of classification (A, B, C, D, etc.). The members of any group have a number of antigenic characters (the O serotype of the organism expressed by numbers 1, 2, 3, 4, etc.) of which at least one is characteristic of the group. The antigenic specificity of the O antigen lies predominantly in the heteropolysaccharide component (often isolated as a lipopolysaccharide), which can be built up of numerous different sugars (heptoses, hexoses, hexosamines, deoxyhexoses, and dideoxyhexoses). Species can be divided into groups according to the distribution of these sugar components and these groups are known as "O chemotypes." It is found that there is a close resemblance between the division into serotypes and that into chemotypes, although often a single chemotype may correspond to more than one serotype. For example, Kauffmann *et al.* (63) showed that 29 different O antigenic serotypes of *Escherichia coli* gave 14 different chemotypes. Cross-reactions will tend to occur between different bacterial families in the enterobacteria, the O antigens of which contain the same chemotype. Thus, the O lipopolysaccharides of cross-reacting *Salmonella* type 35, *E. coli* type 111, and *Arizona* type 20 all have the chemotype—glucosamine, galactose, glucose, and colitose—while *Salmonella* type 38, *E. coli* type 21, and *Arizona* type 16 all have the chemotype —glucosamine, galactosamine, glucose, and galactose (142). The extent of cross-reaction depends on the similarity of the linkages at the end of the polysaccharide chains together with the specificity of antibody production in the animal used. Another example of the value and limitations of the determination of chemotype concerns the occurrence of N-acetyl neuraminic acid in bacterial polysaccharides. This occurs in at least three different antigenic forms which do not cross-react (8): (*a*) The lipopolysaccharides from group Y *Salmonella* where the N-acetyl neuraminic acid is responsible for the specificity of antigen 48 (142). This polysaccharide cross-reacts with the N-acetyl neuraminic acid containing heteropolysaccharide of *Citrobacter freundii*. (*b*) A homopolysaccharide called colominic acid which is possessed

TABLE I

THE SEROTYPES AND CHEMOTYPES OF A NUMBER OF *SALMONELLA* GROUPS [FROM (64)]

Group	Typical Serotype	Chemotype									
		Glucosamine	Heptose	Galactose	Glucose	Mannose	Rhamnose	Abequose	Colitose	Paratose	Tyvelose
A	1, 2, 12	+	+	+	+	+	+	−	−	+	−
B	4, 5, 12	+	+	+	+	+	+	+	−	−	−
C 1	6, 7	+	+	+	+	+	−	−	−	−	−
C 2	6, 8	+	+	+	+	+	+	+	−	−	−
D 1	1, 9, 12	+	+	+	+	+	+	−	−	−	+
O	35	+	+	+	+	+	−	−	+	−	−

only by the K 1 antigen of *E. coli* (6, 7). (*c*) The specific hapten of group C *Neisseria meningitidis* (139) which is also largely composed of units of N-acetyl neuraminic acid.

A comparison between serotypes and chemotypes in *Salmonella* shows a similar relationship. The sugar components of the O antigenic lipopolysaccharides of 93 salmonella cultures belonging to 36 O groups or subgroups and comprising 90 different serotypes, have been analysed by Kauffmann *et al.* (64). In all, 16 different chemotypes were found and all of the O antigens belonging to a single O group or subgroup had identical chemotypes. Glucosamine, heptose(s), galactose, and glucose were present in all chemotypes. Up to three other sugars may also occur, the relatively lipophilic 6-deoxyhexoses (fucose and rhamnose) and the 3, 6-dideoxyhexoses (abequose, colitose, paratose, and tyvelose) being particularly common in the more complicated chemotypes. The results for some of the *Salmonella* O groups which have been most extensively analysed are given in Table I. The 3, 6-dideoxyhexoses are particularly important as the antigenic determinants of O group specificity (144). They occur as end-groups, being the first sugars to be liberated during acid hydrolysis (95, 143); they also inhibit specifically and at low concentration the reaction between the O antigen and its antibody (122). Other sugars inhibit the reactions of some serotypes, but are usually less specific. Lüderitz *et al.* (77) have obtained further evidence of the importance of dideoxyhexoses as end-groups by producing antibodies against artificial antigens prepared by coupling diazotised *p*-aminophenyl colitopyranosides to proteins. These antibodies reacted with certain colitose-containing polysaccharides (those of cross-reacting *Salmonella* O 35, *E. coli* O 111, and *Arizona* O 20), suggesting that the structural arrangement of the terminally linked colitose was similar to that of colitose in the artificial anti-

gens. On the other hand, the terminally bound colitose in the polysaccharides of cross-reacting *Salmonella* O 50, *E. coli* O 55, and *Arizona* O 9 did not react, suggesting that a different type of colitose linkage was involved, a suggestion reinforced by the lack of cross-reaction between the two groups.

Inhibition studies by Staub *et al.* (122) have identified the main determinant of a number of antigens and it is probable that this determinant is always a terminal sugar. The results for a variety of salmonella groups are shown in Figure 1. More recent evidence reported by Kotelko, Staub & Tinelli (66) suggests that antigen 5 in *Salmonella typhimurium* (O serotype 4, 5, 12) is determined by the presence of terminal O-acetyl galactose groups. The importance of acetyl groups in determining specificity has also been shown for the Vi antigen where the differences between the antigenicity of the different species may be tied to the degree of acetylation (145). The evidence suggests that all the determinants reside in the same molecule. Precipitation of the salmonella O lipopolysaccharides by antibodies specific for one or more of the serotypes present always brings down the component sugars in the same relative proportions (76, 121). This multivalent nature of the antigen is presumably a reflection of the branched nature of the polysaccharide chain.

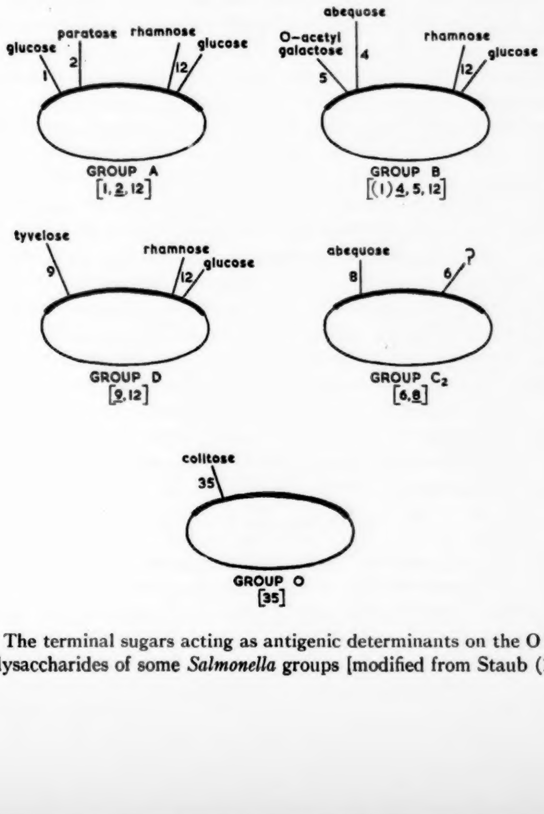

FIG. 1. The terminal sugars acting as antigenic determinants on the O antigenic polysaccharides of some *Salmonella* groups [modified from Staub (120)].

As stated previously, the linkage of the terminal sugar and the sugars adjacent to the terminal one are also important in determining immunological specificity. The probable nature of some of these adjacent sugars can be determined by a chemical and immunological study of oligosaccharides produced by partial hydrolysis of the polysaccharide. Antigens 1 and 12 in *Salmonella* both have glucose as the terminal dominant sugar, but differ in the linkage onto the adjacent sugars. In antigen 1, the probable sequence is

$$\text{glucose} \overset{1\text{–}3}{\underset{1\text{–}6}{\text{or}}} \text{galactose-mannose-rhamnose-} \ (127),$$

while in antigen 12 it is glucose 1:4 galactose-mannose-rhamnose- (132). In antigen 9, the terminal grouping is thought to be tyvelose 1–3 mannose 1- (131).

Although a substantial start has been made towards the determination of the chemical basis of the antigenic specificity of salmonella O polysaccharides, the position with regard to naturally occurring non-conjugated proteins is less satisfactory. By far the majority of proteins contain a selection of the same 20 amino acids joined primarily by peptide linkages. Therefore, antigenic specificity could hardly be normally due to a unique component or a unique type of linkage as often occurs in polysaccharides. Further, there is no evidence of the importance of the end-groups in proteins for antigenic specificity. Instead, there are assumed to be numerous areas on the surface of the protein molecule which act as antigenic determinants, antibodies being specific for a particular surface arrangement of amino acids. As with polysaccharides, a pure protein will probably normally produce a range of different antibodies specific for different parts of the surface. Thus, the minimal number for the "valence" of ovalbumin is 5, for diphtheria toxin 8 and for thyroglobulin 70 [references in (46)]. Unless these "valences" are all caused by the same chemical grouping on the antigen surface, we must assume that each protein can produce more than one type of antibody. This can also be seen in flagellar H antigens, where Nakaya, Uchida & Fukumi (89) have shown that the antigenic reactivities of *Salmonella enteriditis* flagella denoted by the symbols "g" and "m" are both borne on each flagellum and it is assumed on each molecule of flagellin. A similar situation has been described by Finger (35) for the protein immobilisation antigens of *Paramecium aurelia* in which it is again found that the different antigenic groupings characteristic of a given serotype are borne on a single molecule. On the other hand, Gard, Heller & Weibull (36) found, by agar-gel diffusion tests, two independent antigens in flagella of *Proteus* X19H.

THE SYNTHESIS OF ANTIGENS

It is necessary to consider briefly antigen synthesis at this stage, since variation in antigenic specificity is inevitably the result of alterations in the synthetic mechanisms of the cell. Loss or gain of an antigen may be caused by loss or gain of the ability to synthesise a complete polysaccharide or poly-

peptide molecule, or it may simply involve one particular antigenic determinant on the molecule. A change in antigenic specificity (i.e., a loss coupled with a gain) may be due to the uncovering of a new antigenic determinant, either by loss of the ability to synthesise an entire antigenic molecule which normally occludes another antigen lying under it, or by an exposure of a new antigen determinant on a molecule by a loss or refolding of part of that molecule. Alternatively, a change may occur by an exchange in the ability to synthesise two quite different antigen molecules.

The synthesis of polymeric antigens can be thought of as occurring in two stages: (*a*) The provision of monomeric subunits in a form suitable for polymerisation (usually a combination with some carrier molecule), and (*b*) the polymerisation of these units with the liberation of free carrier molecules. In theory, specificity in either of these stages can give rise to the antigenic specificity of the final product.

The Provision of Monomeric Units

Amino acids in polypeptide synthesis.—According to current ideas of protein (heteropolypeptide) synthesis, the amino acid monomers are activated enzymically and transferred onto specific soluble ribonucleic acid molecules which act as carriers. Since the amino acid sequence of a protein is probably invariable and since a selection of the same 20 amino acids probably occurs in all typical proteins, a change in genotype resulting in a change in the ability to produce a particular amino acid donor will affect protein synthesis in general. A complete loss in such a synthetic ability will be lethal to the cell. Consequently, an alteration in the ability to produce the amino acid donors would not result in antigenic variation.

The only exception to this rule may be in the unusual amino acids known to occur extracellularly in microorganisms. One of the most interesting examples is in the recent discovery by Ambler & Rees (1) of N-methyl lysine in the flagellin of some *Salmonella* strains. The ability to gain or lose the N-methyl lysine component of flagellin is controlled by a gene which is separable from the regions of the chromosome governing flagellin synthesis in general (see p. 287). This gene may be responsible for the production of an enzyme required for the synthesis of the N-methyl lysine donor (e.g., an enzyme-methylating lysine), although it is not known at present whether the lysine is methylated before or after combination into flagellin. Further, it remains to be explained how N-methyl lysine is present only in flagellin and not in other bacterial proteins. Is there something unusual in the mechanism of flagellin synthesis? Inability to synthesise the other unusual amino acids of the cell wall and capsule such as diaminopimelic acid or the D-amino acids may also result in antigenic variation although it must be realised that these compounds occur in atypical peptides which are normally occluded antigenically and which are probably synthesised by a quite different route than that of protein synthesis in general.

Sugars in polysaccharide synthesis.—There is much evidence for the

utilisation of nucleotide-phosphate-sugar compounds as the glycoside donors in polysaccharide synthesis. These compounds act in a dual role by providing intermediates for sugar transformation reactions as well as acting as glycoside donors. In the synthesis of homopolysaccharides, uridine compounds seem to be usually involved, e.g., with the appropriate uridine diphosphate sugars in the synthesis of cellulose (39), glycogen (71), chitin (40), β 1-3-glucan (34), hyaluronic acid (86), and the type III pneumococcal polysaccharide (114). The last two examples can be considered as simple polymers of a disaccharide unit. Studies of several strains of pneumococcus by Smith *et al.* (113, 115, 116) have demonstrated the probable importance of uridine diphosphate sugars in the metabolism of the hexose and uronic acid components occurring in some of the type-specific polysaccharides. However, uridine compounds are probably not so universal as was originally thought. Guanosine diphosphate derivatives of glucose, mannose, fucose and colitose (38, 43, 96), and thymidine diphosphate derivatives of rhamnose and other sugars (92, 129) have been found in bacteria. Related to these are the cytidine diphosphate derivatives of glycerol and ribitol which are possibly concerned in the synthesis of teichoic acids and polyglycerophosphate (2, 81).

Since many sugars seem to occur only in specific polysaccharide antigens, a mutation resulting in a change in the ability to synthesise the glycoside donor will specifically affect the particular antigen. It is probable that many of the mutations resulting in the variation in polysaccharide antigens are due to a change in the ability to synthesise the appropriate glycoside donors. Examples of this will be seen later during the discussion of changes in the ability to synthesise the type-specific polysaccharides of pneumococci.

The Polymerisation of Monomeric Units

Polymerisation may occur under the action of some type of template system or by reactions catalysed solely by enzymes.

Non-enzymic polymerisation.—Here, specificity is controlled by a system which is non-enzymic and which is conveniently known as a template. According to current views held by molecular biologists, the structure of typical proteins is determined by a coding system provided by the nucleotide sequence of nuclear deoxyribonucleic acid. This is thought to control amino acid sequence, probably through the intermediary formation of specific ribonucleic acid molecules. It is not, however, usually thought that polysaccharides and other non-protein antigens are formed in this way. However, it has been suggested that the specificity of polysaccharide synthesis might also be controlled, at any rate in part, by some sort of template mechanism (146). Although most proteins are synthesised by a non-enzymic polymerisation, it is probable that some typical microbial polypeptides (e.g., the basic cell wall mucopeptide of bacteria or the polyglutamic acid capsular material) are synthesised by an enzyme-catalysed polymerisation.

Enzymic polymerisation.—Here the specificity of the antigen is controlled by the specificity of the polymerising enzymes. Although it is probable

that most surface antigens are synthesised in this way, the enzyme systems have been studied in very few cases, and these the simplest ones. For example, the only cell-free systems synthesising a polysaccharide containing more than one sugar are those for hyaluronic acid (86) and type III pneumococcal polysaccharide (114). However, these are both simple polymers of disaccharide units and in neither case have the enzymes been isolated and studied in a reasonably pure state. We have no evidence of the mode of synthesis of the more complex branched polysaccharides containing many different sugar units which are the commonest antigens in microorganisms. If it is enzymic, then numerous specific enzymes must be involved to give a structure which may well have an invariable constitution under different environmental conditions (147). Antigenic variation can result from a loss or gain in the ability to synthesise one of these polymerising enzymes. This may result in loss or gain of the ability to synthesise the entire polysaccharide molecule as often occurs in proteins. Often, however, it may result in the production of a changed polysaccharide, possibly with different terminal antigenic groupings. An example is seen in the group-specific polysaccharides of a group A streptococcus and its variant (V); both contained rhamnose and N-acetyl glucosamine, but in different proportions (82). Acid or specific enzymic hydrolysis was used to determine the reason for the quite different antigenic specificity of the two polysaccharides and it was found that in group A polysaccharide the determinants were terminal N-acetyl glucosamine (80). Variation resulted from the removal of these terminal N-acetyl-glucosamine groupings exposing underlying rhamnose groupings which became terminal and were responsible for V activity.

Mention here should be made of variation in the mucopolysaccharide determinants of the blood group antigens A, B, H, and Le^a. Morgan and Watkins (88, 138) suggest that all of the above types are produced by additions onto a common precursor. All these blood group mucopolysaccharides contain L-fucose, D-galactose, D-glucosamine, D-galactosamine, acetyl groups, and 11 to 12 amino acids. Polysaccharides of different types differ in the nature of their terminal sugars, H, A, and B specificities being due, respectively, to terminal L-fucose, N-acetyl glucosamine and D-galactose. The addition of these various specificities onto the precursor molecule is presumed to be controlled by a series of genes which function by bringing about an orderly conversion of the precursor, presumably by the addition of a new terminal sugar together, possibly, with adjacent groups.

The Genetic Basis of Antigenic Variation

If we assume that genes act by controlling the production of specific heteropolypeptide chains containing a selection of the standard range of amino acids, then a consideration of the mechanisms of antigen synthesis makes it reasonable to suppose that a gene may affect antigenic specificity in two ways. First, there may be a "direct effect" on the synthesis of an antigenic heteropolypeptide chain and this would result in a "1:1" relationship

between an antigen molecule and a gene. Secondly, there may be an "indirect effect" wherein a gene acts by controlling the synthesis of a heteropolypeptide which, in its turn, acts as an enzyme catalyzing either synthesis of a monomer donor or polymerisation of the monomers into a polypeptide or polysaccharide antigen. Here a 1:1 gene-antigen relationship would not be expected, since the synthesis of a given antigen would be controlled by more than a single enzyme and therefore, presumably, by more than a single gene. In view of the considerations discussed previously, an "indirect effect" would be expected to occur in the synthesis of polysaccharides and a few "atypical" polypeptides but not with most proteins. Whether the effect is "direct" or "indirect," however, the result might be either a loss in ability to produce a given heteropolypeptide chain or a production of a different heteropolypeptide with altered antigenic (or enzymic) properties.

Other possibilities for an "indirect" effect can be envisaged. First, where an antigen is not synthesised on the surface of the cell, there might be an inability to transfer the antigen from the site of its formation (presumably in the protoplast) to the surface. For example, in flagella the basal granule is assumed to be concerned with the ability of the cell to condense the antigen flagellin into a functional flagellum. A genetic change may affect the functioning of the basal granule rather than flagellin synthesis. Secondly, the antigen may be secreted entirely in a soluble form so that the cell itself is no longer agglutinated by the specific antibody. For example, a mutant has been obtained from a capsulated type 54 *Klebsiella aerogenes* strain which synthesises all of the type 54 polysaccharide as a soluble slime (147). Thirdly, the antigen may be broken down by an enzyme produced by the same cell.

Turning from these theoretical possibilities based on chemical considerations to the actual genetic situation, we find that there is often a 1:1 relationship between genes and antigens. This has been recognised in higher animals for many years from studies with the mammalian blood group antigens [see Haldane (42)] in which it is known that each antigen has its own specific determining gene and that modifying genes or environmental factors are in general not involved. This is not normally the situation with genetically-determined characters other than antigens (apart, of course, from variations in enzymes and other proteins that have been studied recently).

It is true that in many microorganisms the environment has an important influence in determining which out of an array of alternative antigens is formed in a given cell. For example, strains of *Bordetella pertussis* have been shown by Lacey (68) to exist in three "modes" (or phases) depending on the concentration of particular mineral salts in the medium, and in *Pasteurella persis* (94), the various antigenic types which develop are dependent on temperature and pH. Furthermore, there is a clear effect of temperature and other environmental factors on the antigens of *Paramecium aurelia* (see page 290). In some of these examples, the environment may actually induce an antigenic change and this is known to be true for *Paramecium;* in others the environment may act indirectly by selecting a few spontaneous antigenic

variants. But, in all such cases, there is no conflict with the view that the specificity of each and every member of the arrays of antigens which a strain of organisms may produce has its own unique determining gene, and in *Paramecium*, at least, there is definite evidence that this is so.

Only future work will show the generality of the 1:1 gene-antigen relationship. It should be stated that by "gene" we are here referring to the unit of segregation and crossing-over studied in classical genetics where the chromosomal limits and substructure of a locus are not specified with great precision, as they can be with some microorganisms. It is now well-known, especially by microbial geneticists, that there is a concept of a "functional" gene, of finite linear dimensions, within which mutation may occur at many separable sites, each such mutation causing a disturbance in the functioning of the whole locus. The "functional" gene may consist of many "sub-genes" which can be made to show, by special techniques involving immense numbers of progeny, very rare recombination (crossing-over). The 1:1 relationship presumably applies at the level of a "gene" considered as a unit of functional activity and an "antigen" considered as a complex molecule bearing many immunologically distinct groupings. There is at present no evidence for supposing that the 1:1 relationship extends to the finer elements in either the genic or the antigenic hierarchies. Indeed it would be a mistake to draw inferences concerning the one from data based on the other, unless and until some correlation between the two has been established.

The 1:1 gene-antigen relationship is not surprising for protein antigens but it is surprising for polysaccharides if, in fact, synthesis of the latter is controlled by a number of distinct enzymes. Either this "indirect effect" is not true for polysaccharide synthesis or, if it is true, then some additional explanation has to be sought for the apparently unitary nature of the genetic elements for polysaccharide antigens. In this connection it will be recalled that clustering of genes concerned with sequential stages in the synthesis of certain products of intermediary metabolism has been shown to occur in *Salmonella* (25) and something of the sort may exist also for genes concerned with polysaccharide synthesis (see pp. 278–79).

It may be as well at this stage to review the various genetic mechanisms whereby antigenic variations can occur in unicellular organisms. These mechanisms are grouped under four heads, as follows:

Gene mutation.—By this term is meant the alteration of a gene from one form or allele to another, without the participation of genetic material from another cell or organism. Formally, one may distinguish mutations which result in a loss of a gene from those involving a qualitative change but in practice such a distinction may be impossible to establish. Furthermore, one may distinguish between loss of antigenic specificities from gains or replacements but here again such interpretations tend to be subjective, depending on the fineness of the serological analysis.

Recombination.—This may be brought about by replacement of a whole chromosome or a portion (sometimes very small) of a chromosome containing

a given antigen-determining gene, by chromosomal material derived from a different strain of the organism and containing an alternative form (allele) of the gene. This process leads to a recombination of the newly substituted gene with the other genes originally present in the recipient cell. An essential prerequisite is the pairing of homologous chromosome regions and, in the case of recombination between genes in one linkage group, crossing-over. In higher organisms, and here we include the Ciliates, pairing of entire chromosomes occurs and is preceded by fusion of male and female gamete nuclei. In bacteria, however, at least three mechanisms are known whereby genetic material from one cell may be incorporated in another: (*a*) by "mating," involving contact of donor and recipient cells followed by entry into the recipient of whole or part of the donor chromosome; (*b*) by transduction, in which fragments of nuclear material from one bacterium are conveyed into another in phage particles; and (*c*) by transformation where DNA from lysed cells of one strain penetrates into the nuclear region of another.

Recombination may involve intact genes, in which case, of course, the cells receiving a new antigen-determining gene develop an antigen identical with that of the cell from which the gene was derived. Rarely, however, recombination of subgenes within a functionally unitary gene may occur; such "intragenic" recombination might result in the production of an antigen dissimilar from that of either parent, but bearing parts of each. (An example of this is given below for *Salmonella* on p. 287.)

Phase changes.—These are caused by changes affecting the expression of particular genes. To illustrate this, one may consider a culture of cells all containing two different genes *A* and *B*, situated at different chromosomal loci, each gene being capable of controlling the specificity of a particular antigen. In some cells gene *A* may be the operative one and gene *B* present in a latent condition; in other cells the position is reversed, *A* is latent and *B* operative. No mutation or substitution of genes occurs in the change from the first situation to the second. The control of such systems is known to be exerted in *Salmonella* (see p. 285) by other genes, and in *Paramecium* by cytoplasmic factors (see pp. 290–91). Phase changes are characterised by their relatively high frequency, their relatively easy reversibility, and sometimes by their susceptibility to environmental factors, and differ from mutations in all these respects.

Symbionts or episomes.—Here the antigens on the surface of a bacterium may be specifically controlled, not by genes of the bacterium itself, but by something in a phage or other symbiont inside the bacterium (59). The clearest example so far is that of the O antigens of *Salmonella* (see p. 280), some of which are known to be formed only when certain phages are present within the bacteria. By removing or introducing phages, the O antigens can be correspondingly varied. Unambiguous separation of this mechanism from transduction may not always be possible, though the distinction seems to be clear with the *Salmonella* O antigens, for there a lysogenized bacterium may be rid of its phage (and simultaneously of the appropriate antigen) by certain

TABLE II

CHEMOTYPES OF SOME PNEUMOCOCCAL TYPE-SPECIFIC POLYSACCHARIDES
[REFERENCES IN (109, 119)]

	Type											
	I	II	III	IV	V	VI	VII	VIII	IX	XIV	XVIII	XXXIII
Glucose	−	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	−	−	−	−	+	+	+	−	+	−	+
Glucuronic acid	−	+	+	−	−	−	−	+	−	−	−	−
Cellobiuronic acid units	−	−	+	−	−	−	−	+	−	−	−	−
Galacturonic acid	+	−	−	−	−	−	−	−	−	−	−	+
Uronic acid (unidentified)	−	−	−	−	+	−	−	−	+	−	−	−
Amino sugar(s)	+	−	−	+	−	−	+	−	+	+	−	−
Rhamnose	−	+	−	−	−	+	+	−	−	−	+	−
Fucose	+	−	−	−	−	−	−	−	−	−	−	−

environmental treatments which have no effect on transduced bacterial genes (134).

These various mechanisms of genetic change affecting antigens occur with varying frequencies and some of them may be very infrequent indeed. Nevertheless, in view of the vast numbers of microorganisms which may accumulate in a culture, and the possibility of a selection of novel antigenic types when antibodies are present, it cannot be doubted that all the mechanisms are of significance in nature. They are, of course, all used as analytical tools by the investigator, as will be seen below.

CAPSULAR ANTIGENS OF PNEUMOCOCCUS

Smooth virulent *Diplococcus pneumoniae* species are divisible into a large number of serological types denoted by Roman numerals, the nature of the type being determined by the chemical nature of the capsular type-specific polysaccharide (TSP). The chemotypes of some of these polysaccharides have been determined and are given in Table II although many of the criteria of identification are not very satisfactory. Although a single TSP is generally looked upon as a single antigen, it is probable that antibody formation is often heterogeneous, being directed to different monosaccharide subunits, (probably end-groups) in the branched TSP molecule. Thus, a study of cross-reactions has shown that antibody against TSP II may be directed primarily against any of the three main monosaccharide components, rhamnose, glucose, or glucuronic acid (44).

As stated previously, it is thought that uridine diphosphate sugars act as important glycoside donors for the synthesis of pneumococcal type-specific polysaccharides. The enzyme systems responsible for the synthesis of uridine diphosphate glucose (UDPG) and uridine diphosphate glucuronic acid (UDPGA) in type II pneumococci and for the synthesis of these sugar deriv-

atives and, in addition, of uridine diphosphate galacturonic acid (UDPGalA) in type I pneumococci have been described (111, 112, 113). They are summarised in Figure 2.

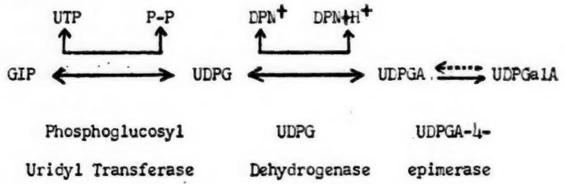

FIG. 2. Uridyl diphosphate sugar metabolism in pneumococci (see text). GIP =glucose-1-phosphate; UTP=uridine triphosphate; P-P=pyrophosphate; DPN =diphosphopyridine nucleotide.

Genetic analysis of antigenic variation in the pneumococcus is theoretically possible, using the phenomenon of transformation as an analytical technique. The work so far accomplished may be considered in two parts: (*a*) that concerned with quantitative variations of capsular material within a given "type," and (*b*) variations from one type to another. These will be taken in order.

Extensive genetic analysis of mutants producing reduced quantities of capsular material has been made. Ephrussi-Taylor (33) studied mutants derived from the normal type III (S III-N) which had reduced ability to synthesise TSP III and were called S III-1, S III-1a, S III-1b, S III-1c, S III-2. The first four were microcapsulate and the fifth formed a capsule of reduced size compared with S III-N. Transforming agents (DNA) from each of the mutants, when applied to pneumococci lacking the ability to synthesise any type-specific polysaccharide (R 36A), yielded transformants identical in TSP III production with the donor of the agent (autogenic transformation). Secondly, when transforming material from one mutant was applied to another mutant, transformants having more TSP III than either "parent" could be obtained (allogenic transformation). Thus,

S III-1 and S III-1c → S III-N
S III-1 and S III-1a → S III-2a

Where S III-N was produced from such recombinations, it was deduced that the two confronted types (S III-1 and S III-1c) were each defective in different portions of the genetic material necessary for development of the normal capsule; where there was an increase in capsular material, but not to the full amount, such as to the type S III-2a mentioned above, it was deduced that the two confronted mutants (S III-1 and S III-1a) were defective in genetic material, some part of which was common to both mutants so that by recombination it would be impossible to reconstitute the normal "wild-type" material. By a series of such recombination experiments, it was

possible to construct a linear "map" of the genetic material concerned with synthesis of the type III capsular material.

In somewhat similar work, MacLeod & Krauss (83) showed that two R mutants derived from S VIII, lacking capsular polysaccharide altogether, could be recombined in transformation experiments to yield fully encapsulated S VIII types. Again, Jackson, MacLeod & Krauss (58) did the same thing with two R mutants of S II. Ravin (101, 102) made a more detailed study of the type III system, working with nine mutants: R_1, R_{11}, R_{27}, R_{6H}, R_x, R_z, S III-1, S III-1c, and S III-2. Transformation tests showed that each of these mutants involved changes in different sites of the genetic material, since "allogenic" transformation could be obtained with any pair, thus

$$R_x \text{ and } R_z \rightarrow \text{S III-N}$$

$$\text{and } R_1 \text{ and } R_{11} \rightarrow \text{S III-2, etc.}$$

From the fact that extracts of S III-2 applied to cells of R_x gave two kinds of transformants, namely S III-N and S III-2, and from other results of similar nature, Ravin concluded that there must be linkage of the different mutation sites, since otherwise two independent transformation events would be required to produce the obtained combinations of types. From the frequencies of various types of recombinant, the following "linkage map" was proposed:

$$—R_1—\text{S III-1—S III-2—}R_{11}—$$

The well-known mutant R 36 (actually a rough mutant of type II) was thought to be a deficiency for the whole region.

The next question to be considered concerns the chemical basis of the reduced ability to produce TSP's. Unfortunately, there is no evidence on the chemical as distinct from the immunological identity of the type-specific polysaccharide from mutants with impaired synthetic ability. In the case of TSP III, however, the simple structure of the molecule makes it probable that the mutants do, in fact, synthesise smaller amounts of an identical polysaccharide. The reason for this reduction in TSP III has been shown to result, in all of those mutants analysed, from a marked reduction in the activity of UDPG dehydrogenase (115). The polymerising systems were assumed to be unchanged. It is probable, therefore, that all of the deficient S III mutants are deficient in UDPG dehydrogenase production, resulting in a reduced level of activity of the enzyme. Similar results have been obtained with mutants that have lost the ability to synthesise capsular TSPI where the mutants fall into two groups (4, 110): the first (S I_2) was deficient in UDPG dehydrogenase while the second (S I_1 and S I_3) was deficient in UDPGA-4-epimerase. The strain S I_2 spontaneously reverted to the normal capsulated state, a change accompanied by increased UDPG dehydrogenase activity. Similarly, restitution of the ability to synthesise normal amounts

of TSP could be obtained by recombination between mutants having deficiencies in the same enzyme (between S I_1 and S I_3 or between S III-1, S III-1b and S IIIc), or between mutants having deficiencies in different enzymes (e.g., S I_1 and S I_2).

Turning to the genetic basis of the difference between one pneumococcal type and another, we find very little precise information. In transformation experiments there is a substitution of the genetic factors controlling formation of one capsular type by those for another; the genetic factor or factors thus behave as a single unit, and it was stated by Ephrussi-Taylor (33) that a given pneumococcus could not possess two capsular agents of different specificity (however, see below). The genetic factors would therefore seem to occupy one and the same chromosomal locus, i.e., to be "allelic." By contrast with the rather common occurrence of mutations from capsulated to non-capsulated forms, mutations from capsulated cells of one type to capsulated cells of another type have never been reported, and this, taken with the probable biochemical complexity of the type variations, makes it seem likely that the genetic differences between the types are also complex. The chance of simultaneous mutation in several genes or genic subunits would be negligibly small.

However, mutation might occur with the loss (or gain) of one antigenic determinant in a complex TSP molecule in an analogous way to that occurring in *Salmonella* O antigens. Such a change has not been described although it is doubtful if it would be observed without the use of special techniques.

The "allelism" of the factors controlling specificity of different types was made doubtful by the discovery by Austrian & Bernheimer (3) of a binary type I–III by transformation of various S III mutants with extracts from S I cells. The binary type was shown to contain two separable polysaccharides within a single cell. It was rather unstable, throwing mutants of pure types I and III; however, in further transformation experiments, using DNA extracts of the binary type I–III to treat type II cells, transformants exhibiting the binary type were obtained, though rarely. Hence, the two genes or gene complexes controlling types I and III specificity are presumably "loosely" linked. Other binary types, such as III–IX, III–V, III–XXV and III–XXXIII have been reported (all containing uronic acid as a component of the capsular polysaccharide).

It should be noted that in all the binary types described, TSP III is always one component. As pointed out by Jackson *et al.* (58), type III cells are also unique among many S strains in not possessing a gene which is concerned in the synthesis of TSP II or a gene which is concerned in the synthesis of TSP VIII. It is possible that these unusual properties of type III strains are due to the very simple chemical constitution of the polysaccharide and hence of the system concerned in its synthesis.

It would seem probable from what has been said previously, that there are some stages in TSP synthesis which are unique to a single type (e.g., the polymerising systems) while others are shared by a whole range of dif-

ferent types (e.g., the synthesis of some glycoside donors). When a rough strain is deficient in a gene or genes controlling the former stages, restoration of the ability to form a particular TSP will depend on transformation by DNA specific to that type while if the deficiency lies in the ability to synthesise a common stage, restoration may occur by transformation using DNA from a variety of different rough strains or smooth types. Transformation in this latter way has been shown to occur in numerous instances. Thus, MacLeod & Krauss (83) transformed various R mutants of type VIII pneumococci with DNA from S cells of types I, II, VII, XIV, and XVIII. Sometimes they obtained a mixture of S VIII cells and S cells of the type used to prepare the DNA extract, sometimes S VIII cells in excess, sometimes the other type in excess. With some combinations, however, only donor S types were obtained, e.g., VIII R-13 treated with DNA from any of the types mentioned above. Several interpretations are possible but it is reasonable to suppose that most mutants of S VIII were lacking in a gene which was common to other types while R-13 was lacking a gene concerned in a reaction specific to TSP VIII synthesis. In further work, Jackson, MacLeod & Krauss (58) studied recombinations using R strains derived from S II cells. All these strains (except R 36) yielded extracts which transformed VIII R to VIII S. An extract of VIII R was capable of transforming II R into a mixture of II S and VIII S. Again, there was evidence of common stages in synthesis between TSP II and TSP VIII.

Further light was thrown on these problems by a study of the binary types described previously (3, 4). The transformation of "deficient" S III cells (i.e., those producing reduced amounts of TSP III) to S I–III by extracts of S I resulted in the production of normal amounts of TSP III in the binary type. It is assumed that when the genetic unit controlling TSP I production is introduced into the deficient S III cell and coexists with S III-determining genes in that cell, the S I unit directs the production of normal amounts of UDPG dehydrogenase and hence the synthesis of both polysaccharides in normal amounts. In agreement with this conclusion is the fact that deficient S III cells can be transformed to S III N by DNA from S I_1 and S I_3 (which contain normal amounts of UDPG dehydrogenase but are deficient in UDPGA-4-epimerase) but not by DNA from S I_2 (deficient in UDPG dehydrogenase but normal in UDPGA-4-epimerase). The S III N strains produced in this way cannot form TSP I because of the mutation affecting the uronic acid epimerase. They are designated S III(I). As expected, in all the types capable of giving binary capsulation (S IX, S XXV and S XXXIII), a uronic acid has been found in their capsule. Indeed, it was found that S V extracts could transform deficient S III to S III–V before the composition of TSP V was known. It was subsequently found, as predicted, that UDPG dehydrogenase and UDPGA were constituents of S V cells and that a uronic acid was a constituent of TSP V [cited in (3)]. Similarly, S XXXIII extracts can transform deficient S III cells to S III–XXXIII and it is found that S XXXIII cells do, in fact, contain a UDPG

dehydrogenase which is involved in the synthesis of the galacturonic acid component of TSP XXXIII (87).

It must be emphasised that binary capsulation is a rare event and that normally transformation comes from a substitution of one genetic element controlling TSP synthesis by a homologous element. Biochemical evidence supports this view. Thus, S I_1 and S II "deficient" cells which contain UDPG dehydrogenase and UDPGA can be transformed to S XIV or S XVIII by extracts of DNA from the appropriate cells. But TSP XIV and TSP XVIII are polysaccharides containing no uronic acid and neither UDPGA nor UDPG dehydrogenase is found in the transformed cells (3). Similarly, S II-deficient cells have been shown to contain the nucleotide UDPrhamnose, the presumed precursor of the rhamnose of TSP II (108). Upon transformation of S II-deficient to S III-deficient cells by the appropriate DNA, UDPrhamnose and UDPGA can no longer be demonstrated in the transformed cells and the activity of UDPG dehydrogenase falls to that typical of S III-deficient cells.

To sum up, it is obvious that at present the precise arrangement of the genetic factors controlling TSP production in the pneumococcus is unknown. However, the specific region in the pneumococcus genome seems to be usually transferred in transformation experiments as a single unit although, in fact, it must consist of a series of separate genes controlling a variety of synthetic reactions, the number of which will presumably vary according to the complexity of the TSP synthesised.

SOMATIC ANTIGENS OF *SALMONELLA*

Nothing has been reported about the control of O antigen specificity in *Salmonella* by bacterial genes. In some instances, however, it is known that presence of a particular component of the O antigen is correlated with a particular symbiotic phage within the bacterium. Iseki & Sakai (56, 57) showed that salmonellas belonging to group E_1 (O antigens 3, 10) were converted to group E_2 (O antigens 3, 15) by exposure to an autolysate of group E_2 organisms containing a temperate phage ϵ15. Uetake, Nakagawa & Akiba (135) extended these observations to a variety of group E *Salmonella* strains. Some of their results are summarised in Figure 3. The following points should be noted: (*a*) Some E_1 strains, when treated by phages from E_2 strains, become converted to types designated "E_1 E_2," in which antigens 10 and 15 coexist. Thus, the addition of an antigen may occur as well as substitution. (*b*) Treatment of cells previously converted to antigens 3, 15, with absorbed antiserum specific for antigen 15 can bring about a reverse change, from antigen 3, 15→3, 10. This reversal is thought to be caused by a selection of cells no longer containing the prophage responsible for antigen 15 formation. Thus, the converted E_2 cells retain a latent ability to produce antigen 10. Possibly the chemical determinants of antigen 15 activity are added on to a side chain with antigen 10 activity. (*c*) *Salmonella chittagong* (O antigens (1), 3, 10, (19)) treated with phage group E_2 strains, may undergo

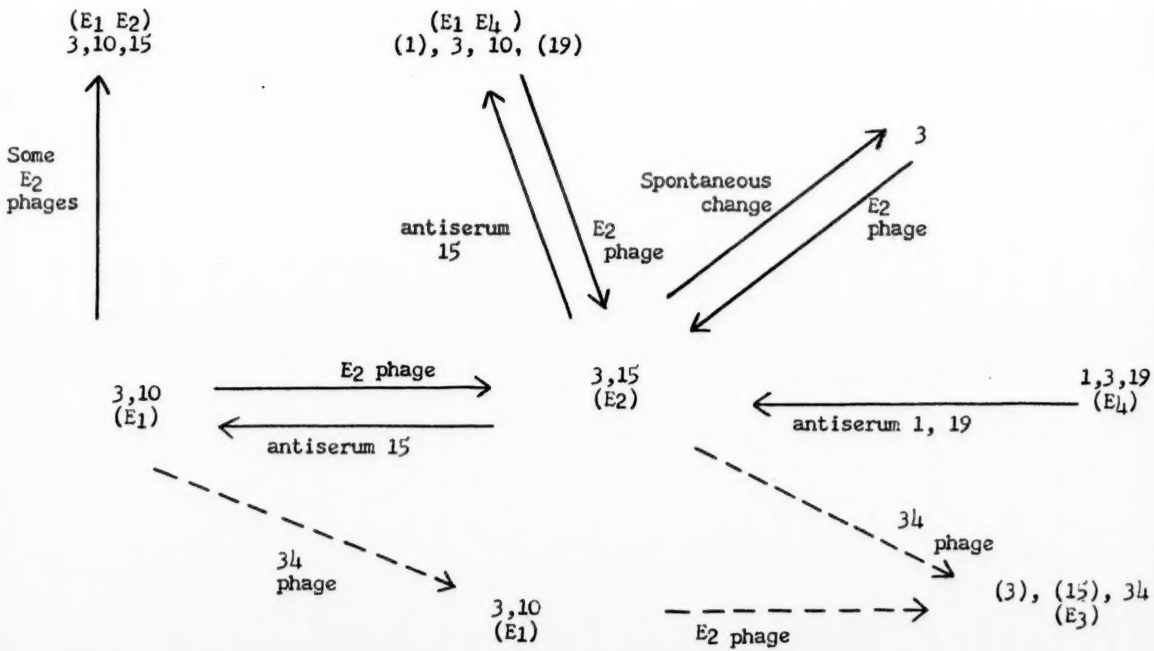

FIG. 3. Some changes in O serotype brought about by temperate phages or antiserum treatment in group E *Salmonella*.
——————→ from Uetake, Nakagawa & Akiba (135);
– – – – – – – – – – – –→ from Uetake & Hagiwara (133).

a complex conversion to group E_2 (O antigens 3, 15). This change was found to be reversible by cultivation in antiserum to antigen 15. (*d*) A comparison of group E_1 and group E_2 strains suggested that naturally occurring group E_2 organisms were the exact lysogenic counterparts of known group E_1 organisms.

Uetake, Luria & Burrous (134) carried out a detailed study of infection of *Salmonella anatum* (O antigens 3, 10) with phage ε15. They showed that antigen 15 appeared in the cells within a few minutes after infection, and that vegetative phage was as effective as prophage in altering the bacterial antigens. The determination of antigen 15 does not require the presence of a stable prophage integrated into the bacterial chromosome.

In addition to the antigenic changes described above, infection with phage ε15 also results in changes of the bacterial surface affecting phage adsorption (134). In the absence of phage ε15, the bacteria can adsorb another phage, C-341; after infection with phage ε15, the bacteria can no longer adsorb phage C-341, but now acquire the ability to adsorb a third phage, ε34. Simultaneously, with the acquisition of antigen 15 and loss of antigen 10, the bacteria gain receptors for phage ε34 and lose those for phage C-341.

Another example of a phage-determined O antigen in *Salmonella* is antigen 34 determined by phage ε34. Uetake & Hagiwara (133) have shown that organisms of subgroup E_3 [O antigens (3), (15), 34] are doubly lysogenic, containing phages ε15 and ε34, and can be produced by infection of group E_2 cells (O antigens 3, 15) with phage ε34. By special techniques, it was possible to infect cells of group E_1 with phage ε34 only (i.e., without simultaneous presence of ε15), and in such cases there was no change in the antigens of the E_1 cells. When such cells containing prophage ε34 only were infected with ε15 phage, antigens 15 and 34 appeared within a few minutes. It was concluded that antigen 15 was a necessary prerequisite for the formation of antigen 34. Antigen 3, as mentioned above, was partially lost together with antigen 15, when 3, 15 cells were converted to (3), (15), 34, but was not considered to be an immediate prerequisite for antigen 34. If we assume that all these antigens are present on the same polysaccharide molecule, it is evident that the presence of a prophage can control the addition of a new antigen on that molecule, presumably in the form of new or altered terminal groups. The partial loss of antigens 3 and 15 in conversion to group E_3 suggests that the determinant of antigen 34 might be added onto terminal groups with 3 and 15 activity. The absence of antigen 34 in cells containing prophage ε34 alone may reflect the lack of some system necessary for the addition of antigen 34 such as the presence of an appropriate glycoside donor which is then supplied by the presence of prophage ε15. It should be noted that groups E_1, E_2, E_3, and E_4 all have the same O chemotype (64).

A final example of the determination of O antigen specificity by a symbiotic phage was described by Iseki & Kashiwagi (55). They found that *Salmonella paratyphi* B (group B) contained several kinds of phage, one of which, ι, governed production of antigen 1. The other phages present had

no effect on this antigen. Phage ι was also shown to control the production of O antigen 1 in salmonellas of groups A and D, but not in groups E, G, and H in which it is assumed that the presence of antigen 1 is determined by bacterial genes rather than prophage genes. Stocker and his colleagues (127) showed that antigen 1 of group E (*Salmonella seftenburg*) and antigen 1 of group B (*Salmonella typhimurium*) both have a common end-group which confers antigen 1 specificity

$$(\text{glucose} \begin{array}{c} 1 \rightarrow 3 \\ \text{or} \\ 1 \rightarrow 6 \end{array} \text{galactose-mannose-}).$$

However, careful tests with absorbed sera showed differences between the examples of antigen 1 in the two groups.

These studies on *Salmonella* O antigens suggest that some determinants can be controlled by the presence of an episome—the prophage. This raises the question as to whether other groupings on the O antigen are determined by the presence of episomal elements. The change from smooth to rough cells provides a possible example. Another example which may be mentioned here is the correlation between phage sensitivity and antigenic specificity in phase II *Shigella sonnei*, wherein acquisition of resistance to phages T_3, T_4, and T_7 is accompanied by a change from a lipopolysaccharide containing glucose, galactose, and glucosamine to one containing only an unidentified hexosamine (41, 60). Although the classical view of S-R variation in the Enterobacteriaceae held that it resulted from the loss of the O antigen and the exposure of an R antigen already present but masked in S forms, recent evidence suggests that it is rather the result of a simplification in the structure of the polysaccharide component of the O antigen. Thus, in *E. coli* 18, the O antigen lipolysaccharide contains galactose, glucose, and rhamnose while the R antigen contains only galactose and glucose (67). In *Salmonella paratyphi* B and *Salmonella typhimurium*, R forms contain glucosamine, a heptose, galactose, and glucose in the lipopolysaccharide while S forms have mannose, rhamnose, and abequose in addition (75). The loss of deoxyhexoses and dideoxyhexoses during S-R mutation seems to be typical (24). It is interesting to speculate on the metabolic significance of this loss of as many as three sugar components of a polysaccharide as the result of a single mutation which occurs at a high rate. Possibly the mutation involves the loss of the ability to synthesise or further metabolise a complex uridine diphosphate compound of the type accumulating during the penicillin inhibition of the synthesis of cell wall mucopeptide. However, sometimes there may be addition of a new sugar as well as a loss. Thus, Davies (23) showed that S-R variation in *Shigella dysenteriae* involved the loss of rhamnose and the gain of aldoheptose.

FLAGELLAR ANTIGENS OF *SALMONELLA*

The specificity of the H antigens of *Salmonella* is considered to be controlled by a protein—flagellin—constituting most of the substance of the

flagellum [see Stocker (125)]. As established by early workers, many strains of *Salmonella* have the potentiality for forming one of two alternative flagellar antigens. Each strain is said to exist in two "phases," the antigens in phase 1 (formerly called the "specific" phase) being denoted by such symbols as a, b, c, d, e, h, i, etc., and those in phase 2 ("group" phase) by 1, 2 e, n, x, etc. Thus, *S. typhimurium* forms antigen i in phase 1 but antigen 1, 2 in phase 2, and has the formula i:1, 2. While some antigens are characteristic of a single phase only, others (e.g., z_{35}) may occur in phase 1 in one strain and phase 2 in another. A minority of strains occur in only a single phase, e.g., *S. typhi* (d:—) or *S. abortus-equi* (—:e, n, x). Very rarely a strain may occur in three phases, e.g., some cultures of *S. goerlitz* (z_{27}:e, h: 1, 2) (72).

The change from one phase to another occurs spontaneously and reversibly, though recently Taylor *et al.* (130) reported some strains in which the change occurred in one direction only, from phase 2 (antigens z_{43}, z_{45} or z_{46}) to phase 1 (antigens g, s, t). Change of phase seems to be uninfluenced by any obvious environmental factors, though growth in the presence of specific antiserum directed against one phase often results in a selection of organisms in the other phase. Stocker (124) found the "mutation rate" from phase 2 to phase 1 in *S. typhimurium* to vary between 10^{-4} and 4.7×10^{-3} in different strains and in the reverse direction between 10^{-5} and 8.6×10^{-4}. The rate of change seemed to be a function solely of the number of cell divisions.

The basic genetic system controlling the flagellar antigens was established by Lederberg and his colleagues, Edwards and Iino, by transduction experiments (69, 70). Two unlinked series of allelic genes, called H_1 and H_2, were found, the H_1 genes determining the specificity of phase 1 antigens and H_2 determining phase 2 antigens. Thus, *S. miami* (a:1, 5) carried the genes H_1^{a} and $H_2^{1,5}$, while *S. abony* (b:e, n, x) carried the genes H_1^{b} and $H_2^{e,n,x}$. By transduction, two new gene combinations were obtained, giving rise to strains with the antigenic formulae a:e, n, x and b:1, 5, and it was found to be the rule that such new combinations involved the substitution of an antigen belonging to one phase by another belonging to the same phase, thus establishing the allelism of the different members of the same phase, and confirming the basic correctness of the phase classification in the Kauffman-White system. An exception to the rule just given has, however, been reported recently by Iino (52). In a transduction experiment using *S. abony* (b:e, n, x) as donor and *S. typhimurium* (i:1, 2) as recipient, an unusual type denoted i:b was obtained. Variation occurred in the new strain from i→b and b→i with the same frequency as did phase variation in the original strain of *S. typhimurium*. It was shown that the gene H_1^{b} from *S. abony* had been transduced into the H_2 locus, replacing $H_2^{1,2}$, since, in subsequent transduction experiments, the b antigen replaced other phase 2 antigens. These results seem to show that the behaviour of these antigen-determining genes is controlled, so far as their phase behaviour is concerned, not by their

own intrinsic nature, but by some other factor, presumably the genetic material in regions adjacent to the normal H_1 and H_2 loci.

The monophasic strains have been shown to be of several different genetic types (69). Thus, *S. abortus-equi*, apparently—:e, n, x, was shown by stringent selection (30) and by transduction (69) to have the potentiality for developing the phase 1 antigen, a, and it was concluded that this strain contained the allele H_1^a but manifestation of the latter was normally prevented because of the action of some other factor (see p. 287). In *S. typhi* (d:—) which lacks a phase 2 antigen, it was found that no H_2 gene could be demonstrated when *S. typhi* was used as either donor or recipient in transduction experiment. This strain does not contain an H_2 gene and cannot incorporate one. *S. paratyphi* B (b:—) may be considered as a third type of monophasic strain. No effective H_2 gene could be transduced from this strain, but when the latter was used as a recipient, incorporation of H_2 genes (e.g., $H_2^{e,n,x}$) was shown to be possible. It might be given the genetic constitution H_1^b H_2^-.

Some monophasic types are characterised by the fact that they develop flagella in one phase only: when in the phase lacking flagella they display only the somatic (O) antigens. Iino (47, 48, 53) found three mutants of *S. typhimurium* lacking flagella in phase 1, but producing typical phase 2 cells. Each of these mutants contained a gene denoted Ah_1^- closely linked to (but separate from) the H_1 locus. However, each of these Ah_1^- genes was shown to occupy a slightly different position on the linkage map, as shown by the results of transduction between different pairs of Ah_1^- mutant, yielding a proportion of Ah_1 recombinants with flagella in phase 1. Another mutant, called Ah_2^-, closely linked to H_2, was found to block development of flagella in phase 2.

We turn now to the question of the subdivision of the H antigens and of the H_1 and H_2 genes. In transduction experiments, the whole group of antigenic components characteristic of a particular phase 1 or phase 2 antigen is transferred intact from donor to recipient. Serological studies of mutant forms, however, have shown that the component parts of these antigens may be separated. Bruner & Edwards (20, 21, 22, 31) earlier found variants of both phase 1 and phase 2 antigens, in which there had been a change in only a part of the specificity of a given antigen, e.g., g, m→g, p; m, t→g, t; g, m, q→g, m in the phase 1 antigens; d, e, h→e, h; d, e, n, z_{15}→e, n, z_{15}; 1, 2→1, 2, 3, etc., in phase 2. Iino (51) has recently found indications that the g, . . . group of phase 1 consists of 8 antigenic subunits (designated f, g, m, s, t, p, q, and u). Mutation at the H_1 locus in *S. typhimurium* (i:1, 2) has been studied by Joys (61), who grew cultures in semisolid medium containing antiphase 2 serum together with sufficient anti-i serum to reduce the rate of spread of phase 1 cells, but not to arrest them completely. In this way mutations resulting in a change in only a part of the i antigen could be obtained by picking out swarms spreading faster through the medium than did the i cells. Nine serological variants of the i antigen were studied,

all of which had gained a new specificity lacking in the 'parent' antigen (i) while retaining the majority of the parent specificities. All of these mutants were shown to arise from changes in the H_1 locus, and, in fact, no mutations of genes at other loci affecting phase 1 antigens were found, confirming the results of Iino (51). Different pairs of the nine mutants were "crossed" by transduction, and some recombinants found which re-established the original "wild type" i antigen. Joys thus established the existence of subunits of the H_1 gene. Using external marker genes (*fla*$^-$) near to the H_1 locus, Joys was able to arrange the mutants in a provisional linear sequence.

Iino (49) described certain mutants of *S. typhimurium* having "curly" flagella, which caused the cells to rotate and aggregate in liquid medium, and prevented swarming on semisolid medium. One mutant produced curly flagella in phase 1 and normal flagella in phase 2, but the antigens in each phase were identical with those of the parent strain. In transduction experiments, the curly gene was found to be inseparable from the H_1 locus. A similar type of mutant was isolated from *S. abortus-equi*, but here the curly flagella occurred only in phase 2, and there was a close linkage with the H_2 locus. Presumably, these curly mutations affect some part of the flagellar protein unconnected with antigenic specificity.

Use has been made of the phase 2 "curly" mutant for more detailed study of the H_2 locus. Transduction from normal e, n, x, to curly 1, 2 was carried out, and of 43 non-curly recombinants, 41 were e, n, x while the remaining two were agglutinated by both anti-e, n, x and anti-1, 2 sera. One of these recombinants carried the antigenic groupings 2, x and part of 1, e and n; the other carried n, x, and part of 1. The results of further experiments of this sort will be awaited with much interest, for they may be hoped to yield information concerning the inter-relations between genic fine structures and the subunits of the antigen molecule.

In the above, we have been concerned with variants causing some modifications in the immunological, and therefore presumably chemical, nature of the flagellar protein. Consideration should also be given to those variants in which formation of the flagella themselves is disturbed.

A common type of mutant is that suppressing formation of flagella in both phases (*fla*$^-$) (128). Some of these mutants are unlinked either to H_1 or H_2, making it possible to obtain strains with particular phase 1 or phase 2 antigens in a latent condition. Some *fla*$^-$ mutants, however, result from genes linked with H_1, forming a cluster near to the H_1 locus. None linked to H_2 have been found as yet. Iino (50) has reported the existence of still further complexity in the segment of genetic material near to H_1. Sixteen non-motile mutants were obtained, of which four were described as "paralysed," i.e., they had flagella but were non-motile. The remaining twelve were *fla*$^-$. Of these, four, when recombined with the others by transduction produced both swarms and trails (i.e., functioning flagella); but six others complemented each other in such a way as to give various incomplete degrees of

restoration to normal flagellation. An attempt was made to map six of these mutant genes in a linear sequence.

Turning now to the problem of the control of the switch from one phase to another, we have to consider what it is which allows the manifestation of an H_1 allele and inhibits the manifestation of an H_2 allele in phase 1 cells, and reverses the situation in phase 2. Lederberg & Iino (70), by various transduction experiments, concluded that, in contrast to the somewhat similar situation in *Paramecium* (see p. 290), the cytoplasm seemed to have played no part in the control of the expression of the H_1 and H_2 genes in *Salmonella*. They found that following transduction of an H_1 allele, the phase of the cells which developed after transduction depended on the phase of the recipient; but following transduction of an H_2 allele the phase of the resulting cell depended on the phase of the donor. Control of phase was thought to be controlled basically by the H_2 locus, which contained genes in an "active" or "inactive" state. However, it is perhaps worth noting that these experiments involved only three strains, *S. typhimurium* (i:1, 2), *S. abony* (b:e, n, x), and *S. heidelberg* (r:1, 2), i.e., three alleles at the H_1 locus and two at the H_2 locus. It is possible that other alleles might behave differently.

Iino (49) found that variability in the stability of phase 2 was controlled by genes at a locus Vh_2 closely linked to H_2. The gene Vh_2^-, derived from *S. abortus-equi* causes development of a stable phase 2 strain, the allele Vh_2^+ of diphasic strains.

It must be stated that the control of change of "activity" of particular genes is an obscure but highly interesting phenomenon. In the genetics of higher organisms some types of position effect are known which may be relevant here, e.g., those cases in which the activity of a particular gene may be suppressed, or favoured, by translocating other genes into the vicinity. However, if a mechanism of this sort is to be applied to the phase changes in *Salmonella*, it would be necessary to assume a rather frequent and regular translocation of genic material to a position near to the H_2 (and possibly H_1) loci. In the absence of any data, further speculation would seem to be pointless at present.

Finally, mention should be made of the variation in flagellar protein resulting from the presence of an unusual amino acid (N-methyl lysine) (see page 269). Stocker, McDonough & Ambler (126) showed that this variant results from a mutant gene closely linked to H_1, but separable from the latter, and probably lying between H_1 and one of the fla^- mutants. It is interesting to note that both phase 1 and phase 2 flagellar proteins are similarly affected with respect to presence of N-methyl lysine. Seven strains of *S. typhimurium* were NML-positive, four strains of *S. paratyphi B* were NML-negative, the rest positive.

Summing up, *Salmonella* may be said to have two chromosome segments containing elements whose mutation may result in (*a*) change in antigenic specificity; (*b*) change in morphology of flagella; (*c*) complete suppression of development of flagella; (*d*) expression of the antigen-determining genes

(phase properties); and (*e*) presence of an abnormal amino acid. Evidently, there is a large and complex array of genetic subunits, whose collaborative functioning results in the development of the normal flagellum.

FIMBRIAL ANTIGENS

Many bacteria belonging to the *Enterobacteriaceae* are known to possess the structures known as fimbriae (or pili). These filamentous appendages can be seen by electron microscopy to be shorter, thinner, and more numerous than bacterial flagella and have adhesive, haemagglutinating and antigenic properties [see (28)]. Although purified fimbriae give a positive test for protein, they contain only 4.6 per cent total nitrogen. The chemical nature of the other component or components is unknown. Gillies & Duguid (37) prepared pure fimbrial antisera by injection of living fimbriate *Shigella flexneri* into rabbits and absorbing the crude immune serum thus obtained with non-fimbriate organisms from the same strain. Brinton (18) prepared an antiserum from fimbriae of *E. coli* by mechanically detaching the fimbriae, purifying them by electrophoresis and injecting the detached fimbriae. Such antifimbrial antisera agglutinated fimbriate organisms but had no effect on non-fimbriate bacteria of the same strain. Treatment of fimbriate *S. flexneri* with specific antisera resulted in adhesion of the fimbriae as seen in the electron microscope and also inhibited the haemagglutinating activity of the bacteria (37). The fimbrial antigens were found to be quite distinct from all other antigens in the bacterial cell since antisera prepared against non-fimbriate cells did not react with the fimbriae. The antigenic composition of the fimbriae of all the strains of *S. flexneri* tested was the same, irrespective of variations in the O antigens, and *flexneri* fimbriae had some antigenic components in common with the fimbriae of many *E. coli* strains but not *Salmonella* strains.

A characteristic feature of fimbriation is the relatively frequent phase change from fimbriate to non-fimbriate cells and the reverse. It is known that particular environmental conditions favour one or other phase: in general, growth in broth tends to produce the fimbriate phase, growth in agar the non-fimbriate, but the change from one phase to another probably does not occur as a direct response to a change of medium. Duguid & Wilkinson (29), describing fimbrial phase variation in *E. coli*, *S. flexneri*, and *S. typhimurium*, concluded that the change probably involves spontaneous changes in a hereditary determinant followed by environmental selection of the variants. However, nothing is known about this hypothetical hereditary determinant.

In addition to the phase variation between fimbriate and non-fimbriate forms, permanently non-fimbriate mutants are also known. In *E. coli*, Maccacaro, Colombo & Dinardo (78) found one such mutant to result from a gene *fim*$^-$ situated in the linkage map close to the *thr-leu* region, and the locus has now been established more exactly between *thi* and *thr* (79). Further, several other variants have been found, e.g., one called *fim* σ^+, which does not show fimbrial phase variation, but occasionally and spontaneously

gives rise to a permanently non-fimbriate form called *fim* σ^-. The genetic factors responsible for the variants *fim* σ^+ and *fim* σ^- are not clear, but there is some evidence for the existence of a factor distinct from, but closely linked to, *fim*.

Some studies have been made on the transfer of fimbriation from *E. coli* to a permanently non-fimbriate form of *Salmonella typhi* (P^-, equivalent to *fim*$^-$ in the above-described work) (19). Following mating of these two species, using *E. coli* as donor, recombinants were obtained having most of the biochemical and antigenic properties of *S. typhi*, but bearing fimbriae. Furthermore, the antigenic properties of the transferred fimbriae were those of the *E. coli* donor.

IMMOBILIZATION ANTIGENS OF *PARAMECIUM AURELIA*

The surface structures of *Paramecium aurelia* are covered with substances called immobilisation antigens. Treatment of the living organisms with dilute homologous antiserum produces a characteristic immobilisation reaction due to clumping of the cilia at their tips. The antigens were formerly called "ciliary" but they have been shown by studies with fluorescein-coupled antibodies not to comprise the structural elements of the cilia. In any case, they are also situated on the pellicle, as shown by differential centrifugation of homogenates (99). The immobilization antigens, whether on living paramecia or in homogenates or in solution, have the property of removing homologous antibody from solutions, and this can be used quantitatively to estimate the amount of antigen present in an extract (9, 16). Another method which has been successfully exploited for quantitative studies of the immobilization antigens is the diffusion of antigen-antibody precipitate in agar-gel (35, 99).

Extensive studies of the genetics of antigen variation in *P. aurelia* have been made by Sonneborn, Beale, and others, but as a number of reviews have been published (11, 12), the details will not be repeated here. Because of the ease with which genetic analysis can be made with this organism, substantial progress has been made in the elucidation of the various factors controlling antigenic variation, a brief account of which follows.

A given stock[4] of paramecia may give rise to individuals bearing one of a series of different immobilization antigens. The maximum number of such serotypes[5] which may occur in a single stock is, at present, twelve (84), but undoubtedly more could be found by further work. Usually the different serotypes of a stock are serologically unrelated, but occasionally cross-

[4] A stock of *P. aurelia* is a culture of organisms all derived from a single individual collected from the wild. They are designated by Arabic numerals, e.g., stock 51, stock 60, etc.

[5] A serotype in *P. aurelia* refers to a group of organisms bearing a particular immobilisation antigen, designated by capital letters, A, B, C, D, etc. Usually the serotype letter is prefixed by the stock number, e.g., 51A.

reactions are found, e.g., between 28G and 28E of syngen 2[6] (5). Indeed, Margolin (84) found in stock 172 a serotype called P which cross-reacted with antisera against no fewer than eight other serotypes. Intrastock antigenic variation was shown by Sonneborn & LeSuer (118) to be based on changes in the cytoplasm, and not by gene mutation or substitution. In a general way, it is a similar phenomenon to the phase change exhibited by the *Salmonella* H antigens.

In *Paramecium aurelia*, by contrast with *Salmonella*, transformation from one type to another may be deliberately induced by certain environmental treatments, such as by changing the temperature. Thus, in syngen 1, many stocks form a low temperature serotype (S), a medium temperature serotype (G), and a high temperature serotype (D). Of special interest is the fact that treatment of paramecia with weak doses of homologous antiserum in some cases brings about such changes of serotype within a stock, e.g., from 51D to 51B in syngen 4, and it has been clearly shown that the mechanism here does not involve selection of a few mutants originally present. It must be added, however, that not all serotypes respond to treatment in this way; sometimes the organisms, after being immobilized, may recover and continue to exist in the same serotype as before. The various factors involved in these experiments have been discussed by Beale (13).

The effect of the genes on antigenic specificity in *P. aurelia* was shown by comparing corresponding serotypes in different stocks and making the appropriate crosses. Thus, it was shown that there were series of multiple alleles at several unlinked loci concerned with antigenic specificity (10, 11, 117). In syngens 2, 4, and 8, the variation produced by the substitution of a gene by another allele at the same locus is usually slight. For example, in syngen 4, the antigenic types 51A and 29A controlled by the genes A^{51} and A^{29} show a high degree of serological cross-reaction. In other syngens (1, 3, and 9), however, the allelic types may be quite distinct when tested by the immobilization reaction. For example, in syngen 1, types 60D and 90D (controlled by alleles D^{60} and D^{90}, respectively) show practically no cross-reaction and were given the same letter only because of the genetic relationship. Later, however, it was shown by Bishop (16) that these types were after all serologically related when other immunological tests (agar-gel diffusion, serum blocking) were applied. It may be stated as a rule that the antigenic variation produced by the substitution of one gene by an allele at the same locus is of a slighter nature than the change of type produced by a cytoplasmic change.

Study of combined genic and cytoplasmic variation (10) has shown that every stock of *P. aurelia* can exist in a series of cytoplasmic states which act in such a way as to inhibit the expression of all the antigen-determining genes except those at one locus. By changing the environment, the cytoplasm can

[6] A syngen (formerly denoted variety) is a group of stocks containing organisms capable of conjugating with each other, and of freely exchanging genes. Some 12 syngens (denoted syngens 1–12) of *P. aurelia* are at present known.

be made to change from one state to another, and this, in its turn, results in a switch in the manifestation of the genes which are effective in controlling the type of antigen formed. The theoretical basis for this system of interaction of genes and cytoplasmic states has been discussed, but no clear solution has been proposed (13). It is possible that the cytoplasmic changes do not involve any loss and gain of particulate determinants, but merely a switch from one steady-state to another, as suggested by Delbrück [see (11)], but there is no evidence directly favouring such a hypothesis.

Unfortunately, it has not been possible to conduct fine structure gene analysis with *P. aurelia.* With respect to mutation work, Reisner (103) showed that following x-ray treatment, a mutant was obtained at the *H* locus in stock 169 (syngen 4) having the phenotypic effect of a 'nul' allele, i.e., the mutant stock could not be induced to pass into the H state under any conditions tried. Beale (13) obtained numerous "nul" types by recombination between pairs of *D* alleles in syngen 1 ($D^{60} \times D^{103}$, $D^{60} \times D^{33}$, $D^{60} \times D^{145}$). The "D-nul" types thus obtained passed over to some other type, such as the G serotype, even under conditions which, in the normal stocks, would have certainly given rise to the D types. Thus, although it seems clear that mutational or recombinational changes may result in inability to synthesize a given antigen, we have no knowledge at present concerning the change in nature of one antigen-determining allele into another, whether by mutation or intralocus recombination. Presumably, an analysis of such changes awaits the development of special techniques (similar to those successfully used with the *Salmonella* H antigens), permitting the selection of variants showing a modification in only a portion of an antigen.

A start has been made with the chemical characterization of the immobilization antigens of *P. aurelia* (15, 97). Preer, working with type 51A (syngen 4) found the molecular weight to be about 250,000 and Bishop (15) found a like value for type 60D of syngen 1. Preer (97, 98) compared the ammonium sulphate solubility of eight different antigens from syngen 4 and two from syngen 2 and found at least four (possibly five) groups on this basis. On the whole, members of a given "solubility group" were serologically related. Preer also found that antigens 51D and 51A were inactivated by chymotrypsin and papain, but that of these two antigens only 51D was inactivated by trypsin. Electrophoretic differences have been shown between antigens 60G and 60D in syngen 1 (15, 17), while the four allelic types 60D, 90D, 103D, and 33D were electrophoretically indistinguishable under the conditions used. Steers (123) also found electrophoretic differences between antigens 51A, 51B, or 51D, of syngen 4. As for the allelic variations, the types 60D and 90D in syngen 1, were separated by adsorption and desorption from a column of calcium phosphate (15, 17).

IMMOBILIZATION ANTIGENS OF *TETRAHYMENA PYRIFORMIS*

A start has been made on the inheritance of antigenic characters in this ciliate. It is apparent that the immobilization reaction here is somewhat

different from that found with *Paramecium*. Treatment of *Tetrahymena* with specific antiserum may result in agglutination and formation of a rigid sheath around the organisms, as described by Robertson (104) and confirmed by later workers. Moreover, the external antigens of *Tetrahymena*, unlike those of *Paramecium*, have been shown to fix complement in the presence of homologous antiserum (107).

It has been established that in *Tetrahymena*, as in *Paramecium*, a single strain grown under different conditions can manifest one of several different serotypes even though a cell normally expresses only one serotype at any one time (54, 74, 85). In variety 1,[7] of *Tetrahymena pyriformis*, each of three strains has been found to be capable of giving rise to three different serotypes, a high temperature (H) type, a low temperature (L) type, and a third type (I) formed by growth of the H type in presence of anti-H serum. Various degrees of serological relation were shown between the corresponding types in the three different strains. When the same strains were grown in axenic medium, more complex results were obtained and there were indications that more than a single immobilization antigen could be formed simultaneously on the surface of a given organism.

Loefer & Owen (73) surveyed 78 strains of variety 1 grown in bacterial medium, and found evidence for at least six different H antigenic types, designated Ha, Hb, Hc, Hd, He and Hf. Nanney & Dubert (91) showed that at least four of these types were determined by allelic genes denoted H^{A}, H^{C}, H^{D}, and H^{E}, each one derived from a different inbred strain. Heterozygotes involving pairs of these alleles displayed a curious phenotypic variability: initially, two distinct antigenic specificities could be recognised, but in the course of asexual fission of the hybrids, sublines arose in which only one type of antigen was expressed. Thus, the heterozygote H^{A}/H^{C} at first gave both types, but after about 120 fissions, out of 133 lines only 21 were $H^{A/C}$, 103 were H^{A} and 9 were H^{C}. This was a purely phenotypic effect since by further crosses it could be shown that, at least in the micronuclei, both alleles were present all along. Presumably this unusual phenomenon is related to the complex structure and mode of reproduction of the ciliate macronucleus. In *Tetrahymena pyriformis*, other characters (e.g., mating types) display similar behaviour (90).

Elliott & Byrd (32) reported that the two mating types (I and II) of variety 7 could be distinguished antigenically, though in variety 1, Nanney (90) found that the antigenic and mating type characters segregated quite independently.

EPILOGUE

"It is a capital mistake to theorise before one has data"[8]

In the course of evolution, unicellular organisms have developed a wide antigenic diversity. For this purpose, they have acquired the ability to syn-

[7] A variety in *Tetrahymena pyriformis* equals a syngen of *Paramecium aurelia*.
[8] *The Adventures of Sherlock Holmes*, A. Conan Doyle.

thesise a variety of unusual components not yet found elsewhere in nature, and build them up, together with more normal components, into macromolecular polymers containing a variety of different linkages. Not only do we find large numbers of antigenic types as typified by the extraordinary range of *Salmonella* strains, but we also find that many microorganisms have developed the ability to change rapidly from one antigenic state to another. The variation can be accomplished by a surprising number of different genetic mechanisms. These may be directed at obtaining a rapid change in antigenicity by means of phase changes, highly mutable genes, etc. The question arises as to the reason for this antigenic diversity. It may be related to the high degree of specificity of agents which can destroy or inhibit the multiplication of microorganisms in their natural environment by reacting with chemical groups on the cell surface. There are two obvious examples of such systems. The first is in microbes parasitic on a higher organism in which the host defense mechanisms involve the production of specific antibodies; bacterial variation resulting in a change in antigenic specificity would allow the survival of the variant when the normal form is being eliminated. The second is the result of the emergence of variants resistant to attack by bacteriophage; such variants often have altered adsorption sites on the cell surface which, as we have seen, may result in antigenic variation. However, although these two mechanisms could theoretically account for much of the antigenic diversity of microorganisms, some cases such as phase variation in *Paramecium* are more difficult to explain. At the present time no satisfactory explanation of the great variability of the immobilization antigens in *Paramecium* has been proposed. It is known that antigenic polymorphism is a constant feature of these organisms, even of small isolated populations in nature (100).

We are beginning to get an idea of the mechanisms underlying antigenic variation. Yet there is need for greater coordination of the serological, biochemical, and genetic approaches, particularly when applied to the same systems in the same microorganism. The classical microbe of the biochemists and geneticists is *E. coli*, but we know little about the inheritance of antigens in it. Two recent papers have provided a start in this direction. Schlegel-Oprecht & Ley (106) have initiated a study of the capsular antigens, and Ørskov & Ørskov (93) have found evidence that a previously unknown antigen (f^+) is specific for F^+ and Hfr strains. Although we know something about the chemistry of *Salmonella* O antigens, the mechanism of their synthesis and inheritance is, with the exception of the phage studies referred to previously, virtually unknown. Similarly, little research has been done on the chemical basis of the specificity of *Salmonella* H antigens or of the immobilisation antigens of *Paramecium*. It is to be hoped that future research, by a greater degree of coordination, will make good these deficiencies.

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RECENT EXPERIENCE WITH ANTIVIRAL VACCINES[1]

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Introduction

Antiviral vaccines, and among them their most potent examples, Jenner's smallpox and Pasteur's antirabies preparations, made their appearances long before the discoveries of the respective viruses. After the viruses had been discovered, new vaccines appeared in gradual succession: against yellow fever (1936), influenza (1937), and tick- and mosquito-borne encephalitis (1939 and 1940, respectively). All of these vaccines were created in that period of developing virology in which the use of small laboratory animals and, somewhat later, of chick embryos, were the prevailing methods of accumulating viruses for antiviral preparations. Following the elaboration and wide virological employment of monolayer tissue cultures (1), vaccines were produced against poliomyelitis (2, 3, 4), adenoviral infections (5), dengue (6), and measles (7, 8, 9). At the same time, tissue culturing began to be used for the modernization of old vaccines as, for example, that against tick-borne encephalitis (10).

It may be expected that the method of monolayer tissue culturing will make it possible to obtain, in the near future, vaccines against those viral infections (epidemic hepatitis) whose specific prophylaxis still lacks any practical solution. One can also hope that this method will be instrumental in pointing up approaches to the problem of specific prophylaxis of cancer, although this may prove to be more complicated than we think.

In this review we should like to focus attention on the problems of active immunization against influenza, poliomyelitis, and measles, not only because the vaccines against these infections, especially against the latter two were obtained comparatively recently, but also because of the fact that some issues appeared in the course of elaborating these vaccines and proved to be quite novel to us. They are as follows: spread of vaccine viruses in the community, reversion of vaccine strains, interference between the vaccine and "wild" viruses, et cetera.

Both inactivated and live vaccines have been proposed for the prophylaxis of influenza and poliomyelitis. The above-mentioned issues sprang up in connection with the use of live vaccines, whereas the study of inactivated vaccines demonstrated that the latter's effectiveness is mostly connected with the dosage of the antigens, their purification, the use of adjuvants and the methods of inactivation and stabilization.

[1] The survey of the literature pertaining to this review was concluded in 1960.

[2] The author is indebted to M. I. Brooke for the translation of this chapter from the Russian.

INFLUENZA VACCINES

General statements.—Apart from the usual difficulties which are encountered in the process of elaborating a vaccine against any virus infection (selection of cultivating media, isolation of vaccine strains, standardization of vaccine preparations, etc.), one must deal with two peculiarities of the influenza infection when tackling the problem of active immunization against it; these create additional difficulties in the solution of the problem at hand. What we refer to here are the peculiarities of immunity found in this disease and the variability of influenza viruses demonstrated in the course of the epidemic process.

Immunity in influenza is specific with respect to the type of the disease. In other words, immunity to the A virus does not include immunity to the B virus since the two types of viruses do not have antigenic components in common. The situation is far more complicated with respect to the specificity of immunity within each of the two main serological types of influenza viruses (A and B).

A full-fledged infectious particle of an influenza virus has two antigens: the soluble antigen (S), evidently connected with the inner nucleo-proteid portion of the virus, and the corpuscular antigen (V) connected with the latter's outer protein-enzymic sheath. The soluble and corpuscular antigens can be separated and respective antisera prepared (11). There is evidence pointing to the existence of a third antigenic component (12, 13) which is determined by the species specificity of the cells (tissues) in which the propagation of the virus occurs. However, the significance of this third component in establishing immunity to influenza is not yet clear. Different strains of influenza viruses relate to one and the same serological type, provided there is an S-antigen common to all of them, although their V-antigens may differ essentially.

The specificity of immunity to influenza is largely associated with the V-antigen, whereas the role of the S antigen in the formation of this immunity is less important. This last statement is confirmed, for instance, by the occurrence of anamnestic reactions in influenza reconvalescents in which antibodies become manifest directed against the antigenic structures of the V antigens of earlier infecting virus strains (14, 15, 16). In view of the above, cross-immunity between the variants of one and the same serological type of an influenza virus is only partial. Apart from numerous laboratory experiments, confirmation of the foregoing statement can be found in epidemiological observation of the A-2 influenza pandemic which was preceded by outbreaks of influenza A-1 (17), as well as in the studies of the effectiveness of influenza vaccines used in the years in which types A-1 (18) and A-2 (19) influenza appeared.

In view of the fact that the new antigenic variants of the A and B influenza viruses do not exist as stable varieties—which is the case with the viruses of poliomyelitis, aphthous stomatitis, dengue, and others—but appear and

disappear periodically in a definite chronological succession in the course of the epidemic process, considerable difficulties arise in the selection of vaccine strains. The latter are effective only within a limited period of a few years until changes emerging in the epidemic process lead to the formation of new variants essentially different in their antigenic structure from their predecessors out of which the vaccine strains had been selected.

In the interval since the A (20) and B (21, 22) influenza viruses were first isolated, their antigenic structures have undergone important evolutions. This is especially pronounced in the A viruses. The varieties that were in circulation in the 1930's and known by the standard strain names of WS and PR 8, were replaced in 1946 and 1947 by the A-1 viruses. Later, in 1957, these in turn were supplanted by the A-2 viruses (23). The essential differences in their antigenic pattern are readily apparent from Figure 1. The data presented in the drawing show that the cross-immunity between the extreme members of the evolutional row of A viruses is almost non-existent. However, there are considerable variations in the antigenic pattern also within the confines of one serological subtype, which is evident from the observations of A-1 viruses isolated between 1947 and 1957 (Fig. 2). Alterations of a similar nature in the antigenic pattern are also observed in B viruses, although the rate of occurrence of these alterations is considerably slower (Fig. 3). It is important to point out that the evolution of the antigenic pattern of influenza viruses occurs at the same period of time, actually simultaneously, the world over, since it is closely associated with the state of specific immunity to influenza (12, 13, 23, 24, 25). In this sense, influenza may, we believe, be considered a truly global infection, since today it afflicts the whole of mankind and is not found only in separate; albeit rather large, areas of the globe (26).

A practical question arises from the foregoing: What are the guiding principles in selecting strains for the preparation of influenza vaccines? One definite factor is that these strains should correspond to the major antigenic variants circulating in the population at a given historical period of time. As applied to influenza of the evolutional row A, these strains must relate to the influenza viruses A (of the 1930's and 1940's), A-1 (1947 to 1957), as well as A-2 (1957 and onward), respectively. However, this circumstance in itself is not sufficient. It is desirable that antigenic variants within each of the mentioned major subtypes be renewed as frequently as possible. In practice, this necessitates renewing the composition of vaccine strains, selecting the latter from among the most widespread variants which circulated in the population at the time of the last epidemic.

A second circumstance which must be taken into account in dealing with the problem of active immunization against influenza is connected with the peculiarities of immunity to this infection. It is known that after infection a short-lived, intensive specific immunity is established; this was learned early after the discovery of the virus, in the course of experimental work with ferrets (27). Later, this issue was subjected to more detailed study

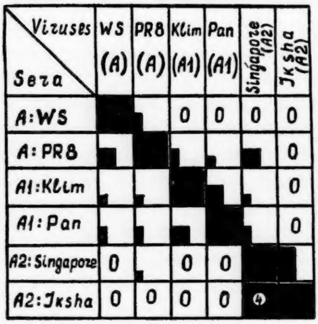

FIG. 1

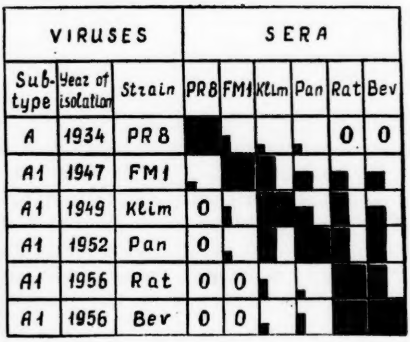

FIG. 2

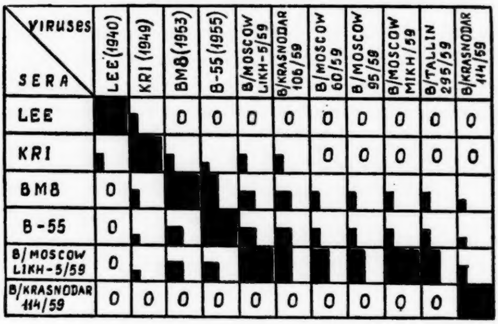

FIG. 3

FIG. 1. Antigenic pattern of influenza viruses, type A (A. S. Gorbunova of our laboratory). ■ ◲ □: degrees of antigenic similarity shown in 1, ½, ¼, etc., of the homologous titer in hemagglutination inhibition test; 0: no similarity; ④ in the square shows that hemagglutination inhibition test with heterologous serum is 4 times higher than with homologous serum.

FIG. 2. Antigenic pattern of A-1 influenza viruses isolated in 1947–1956 (A. S. Gorbunova of our laboratory). For explanation see Figure 1.

FIG. 3. Antigenic relationships between strains of influenza virus B/59 and previously isolated strains (V. M. Stakhanova of our laboratory). For explanation see Figure 1.

in experiments with volunteers (28). A large group of volunteers was immunized intranasally with live attenuated influenza viruses; this was followed by the administration of the same viruses at different time intervals. Apart from determining the levels of antibodies in the blood and nasal cavity, the propagation of the virus in the mucous membrane of the nasopharynx

was taken as the principal criterion of immunity. In another series of observations, similar experiments were performed on influenza convalescents who, at different periods, were given inoculations of the influenza virus previously isolated from them. These experiments demonstrated that convalescents or persons vaccinated with live viruses preserve immunity within the first several months after vaccination or infection of a degree as to prevent the propagation of the viruses in the nasopharynx after a repeated inoculation. However, in four to six months this immunity becomes so weak that an inoculation of the virus is immediately followed by its propagation in the mucous membrane of the nasopharynx. The percentage of persons losing immunity (Fig. 4), as well as the intensity of the propagation of the inoculated virus (measured by virus titers in the nasopharynx and the number of days during which it may be isolated) show an increase within the next few months so that after the lapse of a year or a year and a half, many of the observed subjects have lost their specifically acquired immunity. It should be pointed out that immunity which develops from a natural infection remains for a somewhat longer time than does that acquired from the inoculation of attenuated viruses.

The observations that have so far been carried out allowed us to conclude that a sufficiently intensive immunity follows influenza as to bar any possibility of the propagation of the virus in the organism. This intense degree of

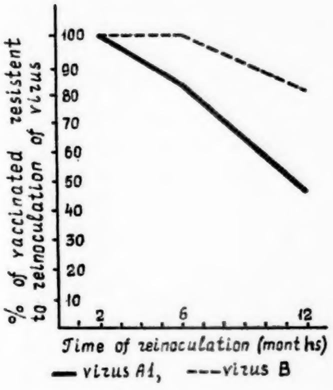

FIG. 4

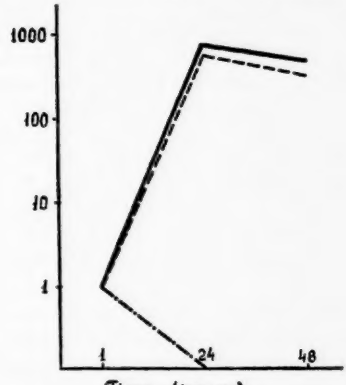

FIG. 5

FIG. 4. Resistance of volunteers vaccinated with A-1 and B viruses intranasally to multiplication of reinoculated viruses after different intervals (L. Y. Zakstelskaya of our laboratory).

FIG. 5. Multiplication of influenza A virus in lungs of mice unvaccinated (———), vaccinated subcutaneously (— — —), and intranasally —·—). Virus titers are expressed in conditional units (one unit is amount of virus one hour after inoculation).

immunity is not preserved for long; evidently it does not remain for more than one year. After the loss of immunity to *infection* there still remains, however, immunity to *disease*, wherein the virus propagates but the usual clinical signs of the disease are absent. The organism remains in this state apparently for one and one-half to two years (probably a little longer in the case of B influenza), following which there is a complete loss of immunity to influenza and the host's reaction to infection is manifested by the usual disease process (24).

The nature of immunity in influenza depends on the routes of the introduction of the virus into the organism, as is well illustrated by studies conducted by Zakstelskaya (28, 29). In experiments with mice immunized with live A-1 virus subcutaneously (first group) and intranasally (second group), and then infected with the same virus intranasally, it was demonstrated that in the first group the virus accumulated in the lungs with almost the same intensity as in the non-immunized controls, whereas no proliferation of virus occurred in the lungs of mice in the second group (Fig. 5). Quite similar data were obtained in experiments on volunteers immunized subcutaneously or intranasally, respectively. These studies demonstrated that the most stable immunity to influenza is established when the virus penetrates into the organism through the natural route, i.e., intranasally. In this case immunity to *infection* is established, which is comprised of humoral immunity (antibodies in the blood), tissue immunity (resistance of respiratory tract epithelium to the propagation of the virus in it), barrier immunity (concentration of antibodies in nasal secretions), and, evidently, a number of other factors involved in building immunity. When administered subcutaneously, the virus predominantly acts as an antigen and not as an infective agent and the immunity thus established to *antigen* is largely humoral. This immunity protects the organism from the disease though it does not prevent the development of infection. All of these factors should be taken into consideration when evaluating the effectiveness of inactivated and live influenza vaccines.

Finally, one should also mention the difficulties that are presented by the objective evaluation of this effectiveness. The variety of etiological forms of diseases clinically similar to influenza (adenovirus, enterovirus, *para*-influenzal and other infections), especially those occurring during intervals between epidemics of influenza, lead to an artificial devaluation of the effectiveness of influenza vaccines because of the presence of these "outsiders" which are, of course, not affected in any way by the vaccines. Therefore, the most reliable data relative to the effectiveness of influenza vaccines can be obtained during epidemic outbreaks when influenza predominates among other numerous influenza-like diseases. Of course, it is possible to detect influenza among other diseases by means of an individual diagnosis of each case occurring in the subjects under observation, but this task proves to be almost impossible to cope with.

Inactivated influenza vaccines.—The use of inactivated vaccines began when Andrewes & Smith (30) and Francis & Magill (31) demonstrated the

possibility of inducing humoral immunity in humans by means of subcutaneous injections of formalinized lung tissue filtrates of influenza-infected mice. There is no need for further expostulation concerning the history of research and improvements of inactivated influenza vaccines. We shall only point out that chick embryo allantoic fluid was used in later stages of this work, and that the virus was concentrated by adsorption to and elution from erythrocytes (32). Less frequently, ultracentrifugation (33) was used. A 0.05 per cent solution of formaldehyde was usually employed for inactivation of the virus, but sometimes workers resorted to exposure to ultraviolet rays. Attempts were made later to use for vaccination preparations of virus which were adsorbed to calcium phosphate (34) or aluminum phosphate (35), or suspended in mineral oil (36). Extensive epidemiologic observations of the effectiveness of inactivated influenza vaccines were carried out by the United States Army Commission on Influenza in 1943–44 (37) and by the Medical Research Council of Great Britain in 1952–53 (38), as well as by many individual authors. In those cases in which the viral strains comprising the vaccines were adequately related to the viruses that caused an epidemic, vaccination proved to be especially effective, bringing about a two to threefold drop in the incidence of influenza among the vaccinated groups. Failures were observed in those years in which the epidemics were caused by new antigenic variants that were not among the components of the vaccines, as was the case, for instance, in the years when A-1 and A-2 viruses appeared. The experience with inactivated influenza vaccines was summed up by the Expert Committee on Respiratory Virus Diseases (23). The Committee recommended inactivated (as well as the live) influenza vaccine as one of the most efficient methods of influenza prophylaxis. The vaccine must be bivalent and must necessarily include viruses A-2 and B, isolated after 1953; it can be administered both sub- and intracutaneously. The vaccine is strongly indicated for use in pregnant women and for persons suffering from diabetes, cardiac, vascular, or pulmonary ailments since in such subjects serious complications or death may frequently follow influenza.

Of late, there has been a noticeable lack of interest among researchers in inactivated influenza vaccines, which may account for the fact that the few reports published on this topic focus largely on details rather than on principal issues of the problem (39 to 43). In all probability, this situation arises from the fact that, on the one hand, the major problems of theory and practical application of inactivated influenza vaccines are considered to be solved and, on the other, that this type of vaccine has never been given a wide practical application. This vaccine has failed to impress the public because, since it fails to protect against the numerous influenza-like diseases caused by other viruses, the inactivated vaccine is wrongly considered to be only moderately effective, or even ineffective. Added to this objection is the unpopular method of administration—via the subcutaneous route. Such are the obstacles to the use of this vaccine in the prophylaxis of influenza, a disease that usually takes a rather mild course.

The inactivated influenza vaccine may find a much wider application in immunizing infants for whom the live vaccine is contraindicated (see below). For obvious reasons, it should be used as a component of the preparation most widely used in childhood, the diphtheria-pertussis-tetanus vaccine. Recently, the author has attempted to make such a preparation (44, 45). The purpose of this research is the attempt to create a basic immunity in children that would protect them against the infection of influenza so dangerous in childhood, and would make it possible to revaccinate them at an older age with the live vaccine.

Live influenza vaccines.—The first attempts to use live influenza virus for active immunization of humans date back to the end of the Thirties (46, 47, 48). Data obtained at that time demonstrated the possibility of inducing antibodies by the subcutaneous or intransal administration of live virus without causing symptomatic infection. Beginning in 1938, Smorodintsev devoted much effort to the elaboration of methods of immunization with live influenza vaccine (49, 50). The first preparations were made from the lungs of infected mice, and later from the allantoic fluid of chick embryos. The vaccine was administered intranasally. Further observations showed a two to threefold drop in the incidence of the disease among vaccinated as compared with non-vaccinated subjects. Beginning in the 1950's, this work has been carried on intensively by Soloviev, Sokolov, Zhdanov *et al.* The research and epidemiologic observations conducted in this field are summed up in the monograph written by Zhdanov, Soloviev & Epshtein (24).

The principles involved in the preparation and application of live influenza vaccines can be formulated as follows (51): (*a*) Influenza vaccines must be administered directly into the upper respiratory passages. (*b*) When selecting strains for the preparation of the vaccines it is necessary to consider the possible antigenic variants within the confines of the two main pathogens of influenza—viruses A and B. (*c*) Immunization of humans must be done with attenuated viruses of sufficient immunogenicity. (*d*) The criteria to be used for the evaluation of the quality of the vaccines are as follows: (*i*) propagation of the virus in the nasopharynx; (*ii*) absence or extreme mildness of morbid symptoms; (*iii*) accumulation of antibodies in the blood and nasal secretions. (*e*) The selection of vaccine strains must be done in such a way that the immunogenic properties inherent in the freshly isolated virus be preserved by means of cultivating the viruses in human tissue. (*f*) Vacuum drying should be used for the preparation of stable vaccines.

These requirements were formulated on the basis of numerous preliminary investigations, during the course of which other problems relating to the use of live influenza vaccines were also solved.

Serial passages of freshly isolated influenza viruses on chick embryos are accompanied by a gradual loss of pathogenicity and immunogenicity, manifested by the loss of abiiity of the viruses to propagate in the human nasopharynx and to produce immunity. It should be pointed out that the loss of pathogenic properties of the viruses occurs rather quickly. After two to three

passages on chick embryos, the virus administered intranasally in large quantities (1 ml. of whole allantoic fluid with a titer in chick embryos of the order of 10^7 to 10^8 ID_{50}) fails to induce disease in the normal subject. The immunogenic properties for humans decrease at a much lower rate in the course of serial passages of influenza viruses on chick embryos; a sharp loss usually occurs after six to eight passages, and this is complete after 12 to 15 passages. These data have been obtained by studying scores of strains some of which, of course, vary. The reports, bearing on the A and B viruses isolated at the beginning of the 1950's, are cited in a number of published works (52, 53, 54). Below is a typical analysis relating to the A-2 influenza virus.

TABLE I

IMMUNOLOGICAL PROPERTIES OF THE A-2 INFLUENZA VIRUS (BERYOZKA STRAIN) AFTER 4 AND 15 PASSAGES ON CHICK EMBRYOS

Number of passages on chick embryos	Names of vaccinated volunteers	Dose of vaccine	Isolation of virus in:				Increase [illegible] of antibodies
			24 hrs.	48 hrs.	72 hrs.	96 hrs.	
4	K-va	0.1 ml. with the titer 10^7 ID_{50} in chick embryos	2/4	4/5	2/5	0/5	8-fold
	S-v		3/4	4/5	0/5	2/5	8-fold
	Sh-v		0/4	2/5	2/4	0/5	4-fold
	A-va		1/4	2/5	1/4	0/5	4-fold
	Kh-va		4/5	0/5	1/5	4/5	16-fold
15	B-v		0/5	0/5	0/5	0/5	0
	Sh-v		0/5	0/5	0/5	0/5	0
	I-n		0/5	1/5	0/5	0/5	0
	K-va		0/5	0/5	0/5	0/5	0
	V-v		1/5	0/5	0/5	0/5	0

The denominator of the fraction is the number of chick embryos inoculated with nasopharyngeal washings in 24, 48, 72, and 96 hrs. after vaccination; the numerator is the number of embryos in which the virus was detected.

Thus, the selection of vaccine strains for the preparation of live influenza vaccine is based on the dissociation of pathogenic and immunogenic (for humans) properties of influenza viruses in the course of serial passages in chick embryos. The strains selected for the preparation of vaccines are of course also investigated for other properties as well (antigenic pattern, sensitivity to inhibitors, biological and hemagglutination titers in chick embryos, etc.).

The inconvenience inherent in this method of selecting vaccine strains is the necessity of testing them on volunteers. Unfortunately, it has so far not been possible to eliminate this limitation, since laboratory tests in animals or tissue cultures fail to show, even approximately, how pathogenic and immunogenic these influenza viruses are for man.

In view of the fact that serial passage in chick embryos leads to the degradation of the immunogenic properties of vaccine strains, a good initial quantity of virus must be available in the serial production of live influenza vaccines in order that large batches of the vaccine may be produced with one additional passage.

However, it is possible to stabilize and strengthen the immunogenic properties of vaccine strains by means of passing them in human tissue cultures (55, 56). This method can be instrumental in increasing the immunogenicity of the viruses undergoing more than 10 serial passages in chick embryos without raising their virulence. Usually, lung tissue or the amnions of aborted fetuses are used for this purpose. The danger of introducing other viruses is virtually eliminated by two or three passages of the strains on chick embryos following passage in human tissues. Only then should the strains be used for the preparation of the vaccines.

The vaccine strains of influenza viruses circulate among immediate contacts of vaccinated individuals (56, 57, 58), although no further spread of the virus (i.e., to contacts of contacts) occurs. Allow us to cite a typical example taken from a work of Slepushkin (58).

TABLE II

ISOLATION OF THE VACCINE STRAIN OF THE A-1 INFLUENZA VIRUS FROM VACCINATED INDIVIDUALS AND CONTACTS

Number of experiments	Vaccinated individuals	Immediate contacts	Secondary contacts
1	9/9	4/5	0/20
4	6/9	4/9	0/7
5	8/8	1/8	0/8

The denominators of the fractions show the number of vaccinated individuals, and the numerators, the number of individuals from whom the virus had been isolated.

The repeated inoculation of the vaccine strain of the virus to some of the individuals cited in Table II was accompanied by its isolation from almost all secondary contacts, and in very few cases, from the vaccinated individuals and immediate contacts. However, even in the immediate contacts, the vaccinating process is of lesser intensity than that seen in the vaccinated subjects themselves, as evidenced by the period required for virus isolation: two to three days are needed for the virus to be isolated from the nasopharynx of immediate contacts, while three to six days are required in vaccinated individuals. Accordingly, the concentration of antibodies in the sera and nasal secretions is higher in vaccinated individuals than in immediate contacts, and is totally absent in secondary contacts.

Vaccination is performed by means of variously designed sprays, depend-

ing on the form of the vaccine used, whether liquid (59) or powder (60). The most widely used immunization scheme consists of the separate administration of A and B viruses with a two-week interval between, although one-stage vaccination with a mixture of both viruses is also a possibility. Reactions to the vaccines are usually moderate, although influenza-like symptoms may develop in certain cases. The reaction-inducing activities of the vaccines vary with the properties of vaccine strains, the state of immunity in the vaccinated individuals and finally, with the time of year. The susceptibility and the reactivity of the vaccinated individuals show a definite rise in the cold season of the year, though this may also be accounted for by the weakening of previously acquired immunity.

All of the foregoing statements are also true of adults or children over six or seven years of age who suffered from A and B influenza at some time. Quite a different picture is observed in children below three or four years of age for whom vaccination with a live virus is practically the first encounter with influenza. This age group reacts strongly even if the dose of the virus is reduced 100 to 1000-fold in comparison with the normal dose of the vaccine administered to adults (0.1 to 1.0 ml. expressed in terms of whole allantoic fluid with the virus titer 10^7 ID_{50}) (61). This peculiarity of the young child precludes the possibility of using live influenza vaccine without first inducing a basic immunity.

The effectiveness of live influenza vaccines has been subjected to innumerable tests which have been summarized in the monograph mentioned earlier (24). Generally speaking, one can say that the effectiveness of such vaccines is very close to that of good specimens of the inactivated ones (23, 24, 62) provided, of course, that the vaccine strains are equatable with the virus responsible for an epidemic. Live vaccines were used during the pandemic of 1957 with positive results (63, 64, 65) as indicated by the rating given by the World Health Organization Expert Committee on Respiratory Virus Diseases (23).

We believe that the possibilities of the inactivated, and especially live influenza vaccines, are far from being exhausted, while the preparations themselves require further improvement and standardization. The latter, however, is extremely difficult because of the lack of a standard pattern in the disease itself. Until chemotherapeutic methods for influenza control are discovered, vaccination will remain the principal method of prophylaxis for this infection.

Measles Vaccines

The problem of obtaining a measles vaccine has hitherto commanded little attention among researchers, and reports on this subject are rather scarce, although the viral nature of measles had been long established and confirmed by many investigations which described the isolation of the measles virus and the study of its biological properties.

The work that has been done during the past 15 to 20 years in the field of measles vaccine research follows these principal lines:

Attempts to use animals for the accumulation and attenuation of the measles virus for its use as a vaccine. Mention should be made in this connection of the investigations conducted by Arakawa (66, 67) who, after lengthy serial cerebral passages of the measles virus in mice, reported on the immunogenic properties of the strains thus obtained and on their evaluation as the result of preliminary experiments in children. No further writings on this particular question appeared, however, and evidently the strains were not tested on a wide epidemiologic basis. Chang Tsen (68) obtained an avirulent measles virus strain through passaging in suckling pigs. An evaluation behavior of this strain was reported after immunologic studies and experiments on children. For the latter, a suspension of living virus was prepared from the organs of suckling pigs at the peak of viremia, and was used for intranasal vaccination. However, trials on a broad scale failed to confirm the effectiveness of the vaccine.

More detailed and extensive investigations along these lines were conducted by Sergiev *et al.* (69, 70, 71), who used the blood of various animals at the peak of viremia as the live measles vaccine. This material was obtained, after a varying number of passages, from rabbits, puppies, and monkeys. Some of the entities of measles infection were reproduced in the vaccinated children (viremia, fever, rash, antibodies in the blood), although the subjects usually contracted the disease when they later came into contact with measles patients.

A number of authors tried to obtain vaccine strains by passaging the measles virus on the allantoic membranes of chick embryos (72, 73). Such cultures of the virus, when administered intranasally or subcutaneously, prevented children from contracting the disease. However, it was later found that the vaccines thus prepared were virulent for children. These results have never been rationally interpreted and contradict the later similar investigations conducted by Ioffe (74) and Zhdanov & Fadeyeva (75), who found that measles viruses, after being passed on the allantoic membranes of chick embryos, are characterized by rather a weak reactivity. The two last authors passed measles virus in human embryo lung explants 6 to 40 times, after which they subjected it to one or two passages in chick embryos and then used the allantoic fluid as the vaccinal material. After the administration of such vaccine intranasally, a mild febrile reaction was observed in some of the children between the eighth and sixteenth days, with leukopenia and sometimes light symptoms of measles as well. Antibodies to the virus developed only in 50 per cent of the vaccinated children. Over 4000 children were vaccinated in a field trial but the vaccine proved to have little effect for although a degree of protection appeared to have been attained in some groups of children, in others all the subjects contracted measles after exposure to infection.

After elaborating the method of establishing monolayer cultures and of adapting measles virus to them (7) attempts were made to prepare a vaccine. The measles virus can be cultivated in many types of epithelial and con-

nective tissue cells, in which it produces cytopathic changes (7, 76, 77, 78) while accumulating in comparatively low titers (usually 2.5 to 4.0 units log ID_{50} when titered on tissue cultures). Of late, a method of virus titration has been elaborated which depends upon plaque formation (79).

Detailed investigations in this direction have been carried out by Enders *et al.*; these have recently been published in a series of works (80 to 87). After it had been subjected to a series of renal cell culture passages, the authors passed an isolated virus of measles (Edmondston strain) in human amnion tissue culture, and only after this last operation adapted the virus to chick embryo tissues. The strain obtained can be considered as fixed since it possesses the following characteristics: when administered subcutaneously to monkeys it causes a mild clinical reaction in 7 to 11 days, the accumulation of antibodies in the blood and ensuing resistance to further inoculations. When injected into monkeys intracerebrally or intracysternally, the virus neither propagates in the brain nor causes viremia.

Observations were made upon 303 children between the ages of one and one-half and ten and one-half years. These children had never contracted measles, as indicated, among other factors, by the absence of antibodies in the blood. After a single subcutaneous immunization of 272 children with vaccine having an infectious titer log ID 1.5 to 2.3 in 0.1 ml. of the culture fluid, 83 per cent of them developed fever, and 48 per cent showed rash. The general state of each child was satisfactory and no catarrhal phenomena were observed. These reactions developed in seven to eight days after the injections and lasted for about three days. Attempts to isolate the virus from the nasopharynx of the vaccinated children failed; the absence of contact transmission of the virus was also proved. A serologic response developed in 95.5 per cent of the vaccinated children and proved rather stable. Upon subsequent exposure, not a single vaccinated child contracted measles, while there was an almost 100 per cent incidence of the disease among the nonvaccinated children in the same institutions and families.

Quite similar results were obtained by Smorodintsev *et al.* (9). Like Enders, he succeeded in isolating a vaccine virus after consecutive adaptation to human amnion and kidney explants and later to fibroblasts of chick embryos. Clinical reactions following subcutaneous and intracutaneous administrations of the preparations were manifested by the onset of fever in six to nine days with rash and, at times, catarrhal symptoms. However, unlike Enders, Smorodintsev succeeded in isolating the virus not only from the blood but also from the mucous membranes of the respiratory passages in some cases. This was true for the most part among children who developed the strongest clinical reactions. There was no further spread of the virus among contacts, as was documented by the absence in them of virus and of serologic conversion. In 24 cases, vaccinated children maintained a long contact with measles patients without, in any instance, contracting the disease.

Both Enders and Smorodintsev tested their strains by administering

them intranasally. The viruses caused vaccinal reactions in only a limited number of vaccinated children. Cutaneous administration of the vaccine yielded negative results.

The investigations conducted by Zhdanov & Fadeyeva (8) followed a somewhat different course. The measles viruses were passed in human amnion tissue cultures (while parallel lines were carried on monkey renal cell cultures). It should be pointed out that the viruses tested underwent only four passages in the human amnion tissue cultures. Preliminary experiments in monkeys demonstrated the presence of immunogenic properties in these strains as tested by challenge with native measles virus present in the blood and mucosa of measles patients. When administered to children, the vaccine elicited much lower reactivity (88) than was seen in the experiments conducted by Enders and Smorodintsev. At the same time, virus was isolated from the nasopharynx not only of vaccinated individuals but also of immediate contacts, although rather irregularly. Further experiences with the vaccinated individuals (81) demonstrated that double or triple subcutaneous vaccination with this preparation creates a good level of immunity. Of the 38 vaccinated children, 24 did not contract measles on later exposure to the disease, while 14 suffered a mitigated form of the infection. Other methods of application of the vaccine (cutaneous and intranasal) failed to create any appreciable immunity to measles.

Thus, the problem of making an effective live vaccine against measles is nearing its solution. The vaccine strains of Enders and Smorodintsev have a number of valuable advantages; they are stable, create a good immunity, and need be administered only once subcutaneously. Their disadvantage is the strong reaction which they evoke, close in intensity to natural measles infection. In this respect, the strains obtained in the laboratory of the author of this review are milder. Their disadvantage is, however, a relatively low immunological effectiveness which necessitates double or triple subcutaneous application for attaining the desired results. It may be expected that in the near future the problem of creating an effective and safe measles vaccine will be solved not only in principle but in practice. At the same time, attempts to obtain inactivated measles vaccines can also be considered as rational. Developed in renal cell cultures of monkeys and inactivated with formaldehyde, measles virus preparations have a moderate immunologic effect (90), which makes reasonable its inclusion in the diphtheria-pertussis-tetanus vaccine.

Poliomyelitis Vaccines

The literature on poliomyelitis vaccines is extremely large and in a brief review one can mention only a few works on this problem.

Inactivated poliomyelitis vaccines.—Early attempts to obtain poliomyelitis vaccines from cerebral tissues of monkeys infected with the poliovirus, with consequent inactivation of the latter by heating, drying, and processing in formaldehyde, phenol, sodium ricinoleate, and aluminum

hydroxide failed, and today have only historical significance (91 to 94). The possibilities of obtaining standard and safe vaccines appeared after the elaboration of methods of cultivating polioviruses in monolayer tissue cultures of humans and monkeys (95, 96, 97).

On the basis of these data, Salk (3) suggested that poliomyelitis vaccine be prepared by cultivating the viruses in the renal epithelial tissue of monkeys with subsequent inactivation in formaldehyde (98). Soon afterward, the vaccine was tested in an epidemiologic study (99), which was followed by its wide application in various countries of the world.

A large scale scale program of prophylactic vaccination with the Salk vaccine in the United States (100, 101, 102) proved to be a success. Within a short time, vast quantities of the vaccine were produced and millions of people were vaccinated; this undoubtedly brought about a sharp drop in the incidence of the disease in that country. Equally successful was the application of the vaccine in other countries of the world (103, 104, 105), especially in those in which all the susceptible population was immunized in a short period of time, as was the case in Denmark and Czechoslovakia (106, 107). The tragic incident that occurred after the use of certain batches of the vaccine which contained active virus and caused disease in the vaccinated groups failed to obliterate its significance as an effective means of polio control although it did provoke a sharply negative public attitude toward the vaccine. Moreover, the incident caused researchers and public health workers to elaborate and use more reliable tests for checking the safety of the vaccine, and to establish temporary international standards for its production and quality control (108, 109).

Greater disappointment in the effectiveness of the vaccine was caused by sudden large outbreaks of polio in the areas of recent mass vaccination against the disease. The most aggravating experiences with postvaccination polio epidemics were reported in Israel and in Detroit, Michigan (110, 111). The analysis of the Detroit epidemics demonstrated that paralytic forms of polio were found most frequently among non-vaccinated people living under unfavorable socio-economic conditions, although the disease also afflicted quite a large group of the vaccinated individuals. Although there were no deaths among the vaccinated subjects (the 22 lethal cases occurred among the non-vaccinated people), nevertheless, of 136 cases of polio among those vaccinated three times or more, 11 per cent of cases terminated in paralysis (70 per cent of paralytic forms were observed among the non-vaccinated individuals). Thus, an intensive immunization campaign failed to prevent an outbreak of poliomyelitis (112).

Salk (113), in his evaluation of the five-year experience with the inactivated poliomyelitis vaccine, suggested the following five reasons for its insufficiently effective action: (*a*) inadequate potency of the batches of vaccine used; (*b*) weak postvaccinal immunologic reaction in some individuals; (*c*) gradual loss of antigenicity of vaccines; (*d*) affliction of the nervous system by the virus, which fails to enter the blood stream and hence misses

the encounter with protective antibodies; (*e*) incorrect diagnoses of diseases with polio-like syndromes caused by other viruses.

Numerous publications of recent years bear on the production as well as the use of inactivated polio vaccine. Special emphasis has been placed on the importance of selecting adequate vaccine strains that will have sufficient antigenic potency (114, 115, 116). The process of inactivating the virus with formaldehyde (117, 118, 119) was thoroughly studied. Tests have been suggested for the evaluation of antigenic potency (120, 121, 122) and for checking the safety (123, 124, 125) of the vaccine. The comparison of different methods of application of formalinized vaccine demonstrates the subcutaneous administration as preferable to the intracutaneous (126). The initial recommendations by Salk regarding the adequacy of triple administration (127, 128) were later revised by him in favor of increasing the number of injections of vaccine in order to achieve an adequate immunity (113). The latest requirements for the polio vaccine (129) demonstrate the results of extensive and intensive research that was conducted in many countries aimed at the improvement of the preparation. The accumulated experience of production and application of the inactivated polio vaccine has been summed up at international symposia and congresses on poliomyelitis (130, 131, 132). In evaluating the experimental materials and practical experience with the Salk vaccine, one should agree with the opinion of the World Health Organization Expert Committee on Poliomyelitis (133) which states that the inactivated vaccine has proved to be a reliable and effective means for poliomyelitis control. Triple immunization with this vaccine decreases the incidence of poliomyelitis among vaccinated individuals as against non-vaccinated ones by 82 to 90 per cent, and the four-step vaccination decreases this incidence by 86 to 96 per cent. Moreover, the course of disease in vaccinated individuals takes a much milder form, which is evidenced by a drop in the percentage of cases of paralysis and the rarity of deaths. Another advantage of this vaccine is that it is applicable in combination with other vaccines, e.g., the diphtheria-pertussis-tetanus vaccine (134, 135, 136).

Simultaneously, the inactivated polio vaccine has disadvantages. One cannot help taking into consideration the fact that even during massive immunization campaigns, large epidemic outbreaks of the disease may occur which afflict a large number of vaccinees. Apart from the above-mentioned outbreaks in Israel and Detroit, there was another large epidemic outbreak of polio in Hungary in 1959, despite the almost total immunization of the population with the Salk vaccine in the previous year (133). Similar situations occurred in some areas in the U.S.S.R. in 1960.

The causes of these phenomena should be sought in the very nature of the immunity produced by the inactivated poliomyelitis vaccine. What was said above of inactivated influenza vaccines, is totally true of the inactivated polio vaccine in the sense that it causes the formation of only humoral immunity, preventing the development of the paralytic form of the disease but failing to prevent the penetration of the polio infection into the in-

testine. For the individual, it means protection from paralysis and nothing more; for the community it offers nothing but the protection of vaccinees from the disease, for vaccination by the Salk method does not affect the spread of polio viruses in the community, since vaccinated individuals are involved in the epidemic process on a par with the non-vaccinated members of the community. The vaccinees, however, face a smaller risk of being afflicted by the paralytic form of the disease. The correctness of this last statement was demonstrated not only experimentally (137) but also by observations of polio epidemics in, for instance, Chicago, Illinois (138) where polio viruses were isolated as frequently from vaccinated and non-vaccinated subjects. These circumstances reduce considerably the value of the inactivated poliomyelitis vaccine and are responsible for the appearance of a new trend in polio research, i.e., the search for a live vaccine. One should not overlook the fact, however, that this search started before the discovery of the inactivated vaccine.

Live poliomyelitis vaccines.—The first attenuated polio virus strains were obtained by Koprowski, Jervis & Norton (139) in 1950 (Strain T, type 2) by cerebral passages in cotton rats. When administered by mouth to 18 non-immune children and adults, propagation of the virus in the intestine was observed together with the accumulation of antibodies in the blood, but with no manifested symptoms. Ten of twelve children proved to be resistant to reinfection with the same virus. The same method was used in obtaining an attenuated strain of the type 1 virus. However, these experiments, repeated by some investigators, did not progress, and more tangible results were obtained after the development of the tissue culture technique, especially after the elaboration of the plaque technique by Dulbecco & Vogt (140), for the isolation of the progeny of single virus particles.

Sabin, Hennessen & Winsser (141) used, for the attenuation of polioviruses, the method of quick passages of massive doses of the virus with the selection of pure lines of low pathogenicity.

The past few years have seen the testing of attenuated Koprowski and Sabin strains on limited groups of children, during the course of which many important issues have been elucidated. It should be pointed out that all strains used were afterward subjected to selection in order to pick out the progeny of single particles of what were, on the whole, heterogeneous populations (142). By 1956, Sabin could already sum up some results of the work done which provided him with the following knowledge (137): The strains obtained by him had lost their neurotropism, or at least part of it, as witnessed by experiments in which the strains were inoculated into monkeys intracerebrally. The dose of 10^4 TCD invariably causes infection of the intestine and the maximum amount of the virus is isolated at the end of the first 10 days, although isolation may be accomplished for as long as three to four months. Antibodies develop in the majority of vaccinated individuals. The viruses isolated from vaccinated individuals do not demonstrate any increase of neurovirulence. In the summarizing review written by Koprowsi

et al. and published somewhat earlier (143), possibilities are stressed of a spread of vaccine viruses among contacts.

By 1958, thousands of people had been immunized with attenuated poliovirus strains, and in summing up the results of these investigations, Dick & Dane (144) pointed out the advantages of the live vaccine over the inactivated one; it is easy to administer (by mouth), cheap to produce, and it is capable of provoking a more lasting immunity, preventing virus reinfection. The inactivated vaccine provides only individual protection from infection and fails to check the spread of "wild" viruses in the community. At the same time, Sabin posed the following problems that had to be solved: The safety of the vaccine for individuals; its safety for the community in the sense of the danger of restoration of neurovirulent properties of the vaccine strain after passage through human intestines; its effectiveness in vaccinated individuals; its effectiveness to the community; and the use and testing of current attenuated strains. The above problems can be supplemented by the problem of interference of vaccine viruses of poliomyelitis when used simultaneously, as well as interference between the latter and the "wild" intestinal viruses, including the causal organisms of poliomyelitis.

Investigations carried out by Smorodintsev *et al.* (145) and by Chumakov and his collaborators (146) in conjunction with Sabin, were of great significance in the further development of live vaccine. Data presented by Smorodintsev include observations of 22,000 children vaccinated with Sabin strains. The observations in question demonstrated the lack of increase in neurovirulence after eight consecutive passages of vaccine strains through susceptible children. Numerous studies have confirmed the safety of the vaccine, have determined the period during which virus is excreted as well as the degree of contact infection, and have revealed a high immunogenicity of vaccine. The same issues were studied by Chumakov who carried out mass immunization in Esthonia (692,000 people), Lithuania (564,000) and Kazakhstan (1,050,000). In his survey written in the middle of 1959 (147) Sabin refers to the mass vaccination campaign carried out in the U.S.S.R., Czechoslovakia, and Mexico as the decisive field trials of his vaccine; by the end of 1959, about 15 million people were immunized in the U.S.S.R. Approximately during the same period, mass immunization with the Koprowski vaccine was carried out in the Congo (148).

In view of the grave importance of the problem of polio control, the rapid development of research into live poliomyelitis vaccine, and the urgency of offering recommendations to governments, the World Health Organization in 1959 and 1960 convened international conferences on live poliomyelitis vaccine (149, 150) during which detailed discussions were organized of the results of numerous experiments and field trials of the vaccine, and the specially convened Expert Committee (1960) worked out the final recommendations (133). At these representative and authoritative meetings and also at the Soviet-American joint discussion on polio (151) at the Scientific Session of the Poliomyelitis Institute of the Academy of

Medical Sciences, U.S.S.R. (151), and at the International Poliomyelitis Congress in Copenhagen (132) all of which took place in 1960—the problems posed by live poliomyelitis vaccine were subject to thorough examination in all their aspects.

Without dwelling at length on the analysis of experimental data and epidemiologic observations of unprecedented objectivity contained in the above-mentioned materials, we shall mention only the principal conclusions that were reached in the Expert Committee's Report (152). At the time the Committee started its work, about 60 million people had been immunized with live polio vaccine, 50 million with Sabin's strains, 7 million with Koprowski's strains, and 2 million with Lederle's strains. The immunization campaign covered large areas in Europe, Asia, Africa, and America; about 40 million people had been vaccinated in the U.S.S.R. by that time.

The Expert Committee on Poliomyelitis elaborated the criteria for estimating the safety of the vaccine, and evaluated field trials and the effectiveness of vaccine application; it also considered the problems of poliovirus vaccine production and various public health aspects of the problem at hand. The conclusion was drawn that the spread of the virus by vaccinated individuals among contacts presents no danger whatsoever contrary to previous apprehensions, and that mass vaccination campaigns demonstrated the absence of any appreciable reversion to neurovirulence of vaccine strains. Special attention was focused on the appraisal of the strains used (Table III).

TABLE III

GRADING OF THE PROPERTIES OF THE VARIOUS VACCINE STRAINS

Desirable properties of vaccine strains	Sabin			Koprowski		Lederle		
	1	2	3	1	3	1	2	3
Low neuropathogenicity for monkeys	a	a	a	b	b	c	c	b
Good immunizing effectiveness	a	a	a*	a	a	a	c	a
Genetic stability on human passage	b	b	c	b	c	b	b	c
Field Trials								
a. Size	More than 50 million			7 million	6 million	approx. 2 million		
b. Age of vaccinated persons	Mainly under 15 years; approximately 500,000 aged 20 or more			Under 15 years	Under 15 years	Mainly under 15 years; at least 200 thousand aged 20 or more		
c. Proportion of triple negatives among vaccinated	10%–14%			—	—	8%–10%		

The ratings a, b and c indicate the order in which strains appear to approach the optimum from the standpoint of possession of desirable properties. a indicates highest rating.

— = information inadequate for a decision.

* Less effective in a trivalent mixture.

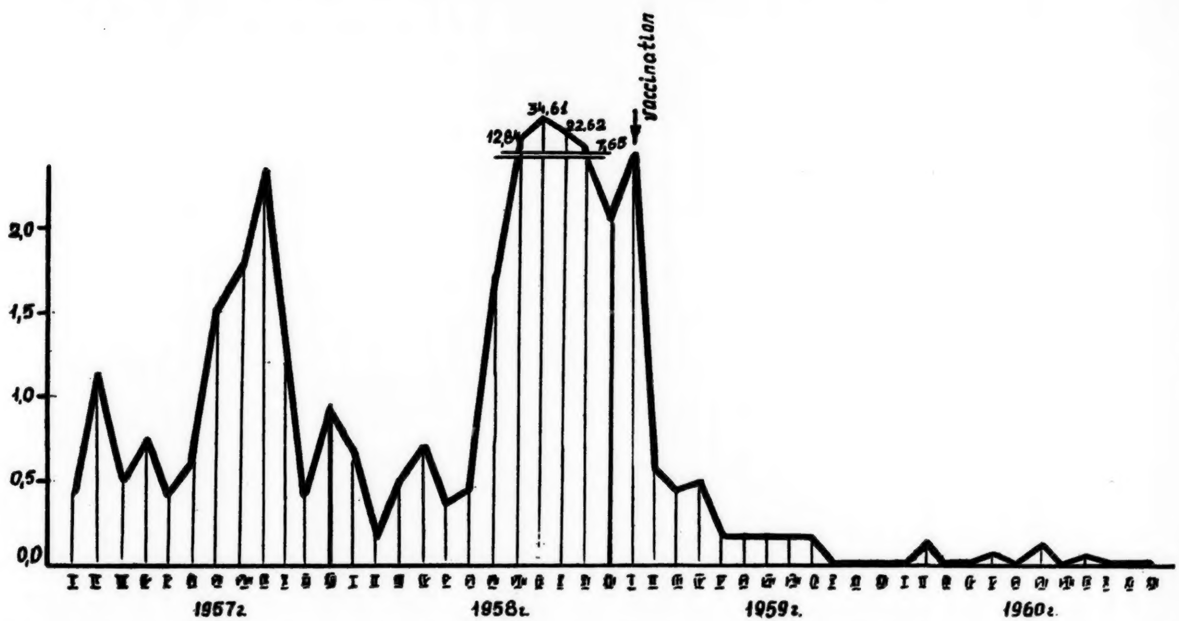

FIG. 6. Incidence of poliomyelitis (all forms) in Estonia in 1957–1960. Cases per 100,000 population in years before and after the use of live poliovirus vaccine in the Baltic Republics of the U.S.S.R.

On the basis of these data the Committee also felt that the overall characteristics of the Sabin type 1 and type 2 strains approach more closely the optimum than do those of the other viruses; the Sabin type 3 strain has undesirable genetic instability and, in view of this fact it has no advantages as compared to the strains of this type developed by other investigators.

At the present time, the application of live polio vaccines has reached an extremely wide scope. The U.S.S.R. completed the first stage of mass immunization against poliomyelitis of its population to age 20, and in some areas of the country, to age 35, with the vaccine prepared from the Sabin strains incorporated in candy. The greater part of vaccination, covering 77 million people, or 35 per cent of the country's total population, was terminated by July 1960.

This massive and simultaneous use of live polio vaccine proved extremely effective, which is confirmed by the results obtained in certain areas as well as in the whole of the country.

In the Baltic Republics of the U.S.S.R., where mass immunization was carried out early in 1959, poliomyelitis was almost completely eradicated for the following two years (Fig. 6). After completing mass vaccination in 1960, a drop in the incidence of the disease was registered in the summer months, when habitually there should be a rise (Fig. 7). In five regions of the country in 1960, immunization was performed not with the live Sabin vaccine, but with Salk's inactivated vaccine, and it was in these particular regions that the usual rise in incidence was observed (Fig. 8). However, even the statistics on these regions failed to exert any substantial influence on the decreasing incidence of the disease throughout the U.S.S.R. (Fig. 9). The experiences with live poliomyelitis vaccine have made it possible to formulate the concept of the total eradication of the disease by means of this vaccine (152).

Conclusion

By paraphrasing the famous Latin quotation, one can say "Habent suum fatum vaccinae."

Research into measles vaccines has proceeded gropingly in the quiet of a few laboratories and to date it is not yet completed. However, the results already achieved bring hope for the practical solution of the problem of vaccination against measles in the near future.

The search for influenza vaccines was intensive, goaded by large-scale epidemics and pandemics of the disease; great hopes were mixed with great disappointments whose bitterness is not yet forgotten. The problem of vaccination against "flu" still awaits its proper solution.

The history of poliomyelitis vaccines has been a short, albeit stormy, one, full of drama and suspense, in the course of which, according to a Russian proverb, "The best became enemy of the good." Polio vaccines, with respect to their popularity and the interest which they command among the people, can probably be compared only with Jenner's smallpox

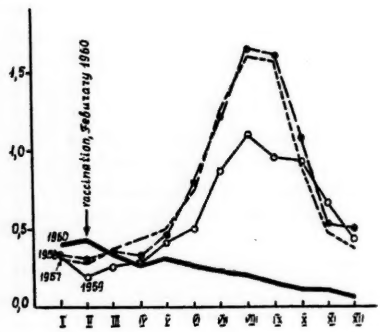

FIG. 7

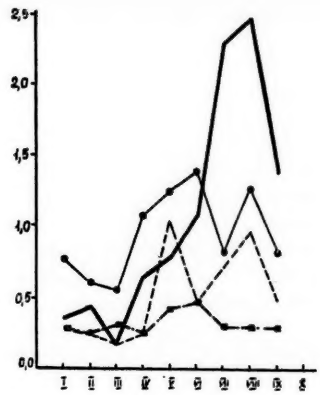

FIG. 8

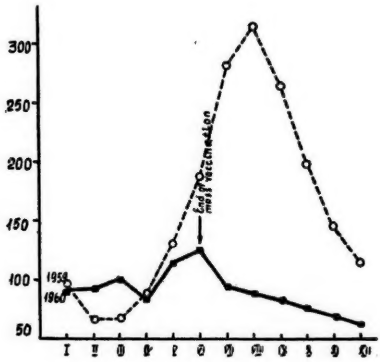

FIG. 9

FIG. 7. Incidence of poliomyelitis (all forms) in Ukraine in 1957–1960. Cases per 100,000 population in years before and after the use of live poliovirus vaccine.

FIG. 8. Incidence of paralytic poliomyelitis in regions where vaccination was performed with Salk vaccine and in regions where Sabin vaccine (RSFSR) was used. Cases per 100,000 population in years before and after the use of live poliovirus vaccine.

——— Krasnodarsky krai
●——● Sverdlovskaya oblast
— — — Leningradskaya oblast
■—·—■ RSFSR

FIG. 9. Incidence of poliomyelitis (all forms) in the U.S.S.R. (including 5 oblasts with Salk vaccination) in 1959 and 1960; incidence in January, 1959, is taken as 100.

and Pasteur's antirabies vaccines, the tempo of life and stormy emotions of our time playing not a small role in it.

We should like to finish this review by expressing a wish that new vaccines, called upon to deliver humanity from numerous and still widespread infections, should bear the stamp of the genius and skill of their authors rather than their whims and characteristics.

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